Univerzita Karlova v Praze
Přírodovědecká fakulta

Studijní program: Biologie
Studijní obor: Buněčná a vývojová biologie

Bc. Radim Žídek

The role of CUP-4 protein in Wnt signalling
Role proteinu CUP-4 ve Wnt signalizaci

Diplomová práce / Diploma thesis

Školitel / Supervisor:
Mgr. Marie Macůrková, Ph.D.

Praha, 2012

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 27.8. 2012

Podpis

I want to dedicate this thesis to Ing. Bořivoj Malec, an amateur entomologist, botanist and traveller, who was my guru through the early (and many of the next) steps in the exciting field of biology.

List of contents

Abstract

Wnt signalling is indispensible for proper development of organisms and maintaining of adult tissue homeostasis. Its disruption often leads to disease. In nematode *Caenorhabditis elegans*, Wnt signalling governs vast array of developmental processes, among others also migration of the Q neuroblasts and their descendants. The sole Wnt acting in this process, EGL-20, triggers the canonical β-catenin Wnt signal transduction pathway in QL but not in QR which leads to QL remaining in the posterior while the QR migrates anteriorly. This represents a useful tool for studying Wnt signalling. Recently, mutation of gene *cup-4* was found to disrupt migration of the QL neuroblast in a small proportion of the mutant population. *cup-4* encodes a ligand-gated ion channel family homologue and it was shown to participate in endocytosis by coelomocytes, specialized phagocytic cells in the *C. elegans* body cavity. Here, I present the results of my effort to determine the place of CUP-4 action in Wnt signalling and to elucidate the mechanism of its function. I found that CUP-4 acts upstream of PRY-1/Axin, which is involved in signal transduction in signal receiving cells, and most probably downstream of adaptin AP2, which is important for recycling of Wnt cargo receptor Wntless (Wls) in Wnt producing cell. *cup-4* also strongly interacts with other genes whose products are essential for proper Wls retrieval – namely with components of the retromer complex genes *vps-29* and *snx-3* and myotubularin phosphoinositide 3-phosphatase gene *mtm-6*. I did not find any notable genetic interaction between *cup-4* and any of the phospholipases C or phosphoinositide 3-kinases tested. Despite the current evidence seems to give a slight edge to the view of CUP-4 functioning in Wnt producing cells, all possibilites still remain and more experiments in the future will be needed to unambiguously resolve CUP-4 mechanism and place of action in Wnt signalling.

Keywords:

Caenorhabditis elegans – development – neuronal migration – Wntless – retromer – endocytosis – phosphoinositides – phospholipases

Abstrakt

Wnt signalizace je nezbytná pro správný vývoj organismů a zachování homeostáze v dospělosti. Její narušení je častou příčinou onemocnění. U háďátka *Caenorhabditis elegans* řídí Wnt signalizace celou paletu vývojových procesů, mezi něž patří také migrace Q neuroblastů a jejich dceřinných buněk. Jediný Wnt, který se podílí na tomto procesu, EGL-20, spouští kanonickou dráhu přenosu Wnt signálu přes β-katenin v QL, ale nikoliv v QR neuroblastu, což vede k tomu, že QL zůstane v zadní části těla, zatímco QR migruje anteriorně. Tato migrace představuje užitečný nástroj pro studium Wnt signalizace. Nedávno bylo zjištěno, že také mutace genu *cup-4* způsobuje poruchu migrace QL neuroblastu u malé části mutantní populace. *cup-4* kóduje protein z rodiny ligandem ovládaných iontových kanálů, který se podílí na endocytóze v coelomocytech, specializovaných fagocytických buňkách v tělní dutině *C. elegans*. V této práci uvádím výsledky mé snahy o umístění CUP-4 do Wnt signalizace a objasnění mechanismu jeho fungování. Zjistil jsem, že CUP-4 je v signální dráze zapotřebí dříve než PRY-1/Axin, který se účastní přenosu signálu v cílových buňkách, a pravděpodobně později než adaptin AP2, který je důležitý pro opětovné využití proteinu Wntless (Wls), receptoru sloužícího k dopravě Wnt proteinu ve Wnt produkujících buňkách. *cup-4* také silně interaguje s dalšími geny, jejichž produkty jsou nezbytné pro fungující koloběh Wls – jmenovitě s geny pro složky retromerového komplexu *vps-29* a *snx-3* a s genem pro myotubularin fosfoinositid 3-fosfatázu *mtm-6*. Nenalezl jsem žádnou významnou genetickou interakci mezi *cup-4* a kteroukoliv z testovaných fosfolipáz C nebo fosfoinositid 3-kinázou. Přestože data v současnosti lehce zvýhodňují hypotézu, podle které CUP-4 funguje ve Wnt-produkující buňce, všechny možnosti zůstávají nadále otevřené a nepochybně bude zapotřebí dalších experimentů, aby se v budoucnu jednoznačně rozřešila otázka mechanismu a místa působení proteinu CUP-4 ve Wnt signalizaci.

Klíčová slova:

Caenorhabditis elegans – vývoj – migrace neuronů – Wntless – retromer – endocytóza – fosfoinositidy – fosfolipázy

List of genes and abbreviations

ALM	anterior lateral MT cell
APC	adenomatous polyposis coli (destruction complex component)
AVM	anterior ventral motoneuron (QR daughter cell)
BSA	bovine serum albumin
CK-Iγ	caseine kinase Iγ
cup-4, CUP-4	coelomocyte uptake defective (referring to *C. elegans* mutant phenotype), ligand-gated ion channel homologue
dpy-20 , DPY-20	dumpy (referring to *C. elegans* mutant phenotype)
dpy-23, DPY-23	dumpy (referring to *C. elegans* mutant phenotype; μ subunit of AP2 adaptor complex in clathrin endycytosis)
DR	dietary restriction
dyn-1, DYN-1	dynamin-like protein
egl-20, EGL-20	egg-laying defective (referring to *C. elegans* mutant phenotype), *C. elegans* long-range acting Wnt protein
ER	endoplasmic reticulum
Fz	Frizzled
gfp, GFP	green fluorescent protein
GSK-3β	glycogen synthase-kinase 3 beta (destruction complex component)
IP3	inositol-1,4,5-trisphosphate
LGI-III	linkage groups one to three (refers to genes located on the same chromosome)
lgc-26, LGC-26	ligand-gated ion channel
LRP5/6	lipoprotein receptor-like protein 5/6
mig-14, MIG-14	migration defective (referring to *C. elegans* mutant phenotype), *C. elegans* orthologue of Wntless, Wnt cargo receptor
mom-1,-2,-3, MOM-1,-2,-3	*C. elegans* orthologs of Porcupin, Wnt and Wls, respectively
mtm-6, MTM-6	myotubularin-related (active phosphoinositide 3-phosphatase)
mtm-9, MTM-9	myotubularin-related (inactive phosphoinositide 3-phosphatase)
nAChR	nicotinic acetylcholine receptor
nlp-1, NLP-1	neuropeptide-like (signalling neuropeptide)

PCP	planar cell polarity (non-canonical Wnt pathway)
PI-3-P	phosphatidylinositol-3-phosphate
PI-3,5-P_2	phosphatidylinositol-3,5-bisphosphate
PI-4,5-P_2	phosphatidylinositol-4,5-bisphosphate
PI-3,4,5-P_3	phosphatidylinositol-3,4,5-trisphosphate
PLM	posterior lateral MT cell
pry-1, PRY-1	*C. elegans* Axin (destruction complex component)
PVM	posterior ventral motoneuron (QL daughter cell)
PX	Phox homology (domain; binds PI-3-P)
QL(.d)	left Q neuroblast (daughter cell) or migration defective phenotype
QR(.d)	right Q neuroblast (daughter cell) or migrationů defrective phenotype
SDS	sodium dodecyl sulfate
sfrp-1, SFRP-1	secreted Frizzled protein (*gene*)
skn-1, SKN-1	skinhead (transcription factor)
snx-3, SNX-3	sorting nexin
Swim	soluble Wingless interacting molecule
TGN	trans-Golgi network
vps-26, VPS-26	vacuolar protein sorting } cargo-selective complex of retromer
vps-29, VPS-29	vacuolar protein sorting
vps-35, VPS-35	vacuolar protein sorting
vps-34, VPS-34	vacuolar protein sorting, PI-3 kinase
Wg	Wingless (*Drosophila* Wnt and the founding member of the family)
Wls	Wntless/Eveness interrupted/Sprinter, Wnt cargo receptor
wt, WT	wild type (refers to one allele, gene or genome/genetic background)

1 Theoretical background

1.1 Wnt signalling

1.1.1 Wnt signalling in development and adult tissue homeostasis

Wnt signalling plays an important role in development and adult tissue homeostasis (Clevers, 2006; Logan and Nusse, 2004); its disruption often leads to cancer (Clevers, 2006; Polakis, 2007). Wnt ligands can spread over distance and act as classical morphogens providing developing tissues with positional information. This information can be used to regulate cell proliferation, migration, differentation, polarization, growth or cell death. Wnts can also act as stem cell factors in maintaining undifferentiated state and developmental potential (Nusse, 2008; Ten Berge et al., 2011; Willert et al., 2003).

1.1.2 Overview of the Wnt signal transduction

There are several mechanisms how the Wnt signal can be further transduced in the receiving cell (summarized in (Kikuchi et al., 2009)). For easier orientation in the following text, I also briefly summarize the Wnt signalling pathway here:

This thesis focuses on the first discovered and best known canonical Wnt signalling pathway. In this pathway, Wnt ligand binds to the Frizzled receptor on the cytoplasmic membrane which, in turn, activates Dishevelled leading to phosphorylation of a Wnt co-receptor LRP5/6 by casein kinase Iα and GSK-3β. This results in an inactivation of the destruction complex composed (besides of both above-mentioned kinases) also of Axin and APC. Without the bound Wnt ligand, the destruction complex phosphorylates β-catenin targeting it to the proteasomal degradation. Upon inactivation of the destruction complex, β-catenin is released and can translocate into the nucleus, where it binds to Tcf/Lef transcription factors and activates target gene expression.

The other activation mechanisms of the Wnt signalling comprise planar cell polarity (PCP) pathway, where the Wnt signal is translated into a rearrangement of the cytoskeleton, the Ca^{2+}/protein kinase C pathway and signalling through an alternative Ryk receptor. Since they are probably not involved in the Wnt signalling that includes CUP-4, they are not discussed in detail.

1.2 Wnt signalling in *Caenorhabditis elegans*

1.2.1 Components of Wnt signalling in *C. elegans*

In nematode *Caenorhabditis elegans*, Wnt signalling is also involved in many developmental processes including antero-posterior regionalization of the body (Harterink et al., 2011a), mesoderm induction (Rocheleau et al., 1997; Thorpe et al., 1997), vulval development , polarization of hypodermal (seam) cells (Whangbo et al., 2000), gonad arm migration and distal tip cell specification (Walston et al., 2006), several neuronal migrations (Silhankova and Korswagen, 2007; Zinovyeva et al., 2008), polarity of neurons and axon guidance (Hilliard and Bargmann, 2006; Prasad and Clark, 2006).

There are five Wnt genes in *C. elegans* (*cwn-1*, *cwn-2* (Shackleford et al., 1993), *lin-44* (Herman et al., 1995), *mom-2* (Thorpe et al., 1997) and *egl-20* (Whangbo and Kenyon, 1999)), and four for Frizzled receptors (*lin-17* (Sawa et al., 1996), *mom-5* (Rocheleau et al., 1997), *cfz-2* (Zinovyeva and Forrester, 2005) and *mig-1* (Pan et al., 2006)). For easier orientation in *C. elegans* nomenclature, proteins showed to participate in EGL-20/Wnt signalling and their mammalian or *Drosophila* counterparts are listed in Table 1. This table is partly based on the one published in (Korswagen, 2002) but it focuses purely on EGL-20/Wnt dependent migration of the Q neuroblasts. Moreover, it is updated and contains additional proteins identified in the past ten years to function mainly in the secretion of EGL-20/Wnt signal protein. Proteins are ordered by their place of action in Wnt signalling from posttranslational modifications of EGL-20/Wnt through its secretion to the events in responding cell including target gene *mab-5*. I have included MOM-1/Porcupine in the list as it is likely to participate in EGL-20 modification, although this has not been formally proven yet.

1.2.2 Developmental events guided by Wnts

The earliest event regulated by Wnt signalling is polarization of the EMS blastomere of a 4-cell stage embryo by its neigbouring blastomere P_2 which produces Wnt MOM-2. MOM-2 binds to receptors on the EMS blastomere and triggers a non-canonical Wnt signalling pathway. As a result, mitotic spindle or EMS reorients so the division plane is located between two poles of anterior-posteriorly polarized cell. E blastomere eventually gives rise to entoderm (Rocheleau et al., 1997; Thorpe et al., 1997).

Table 1 **Components of EGL-20/Wnt signalling as described in literature**

Component of Wnt signalling	***C. elegans* homologue**	**Reference**
Porcupine	MOM-1	-
Wntless	MIG-14	(Rocheleau et al., 1997; Thorpe et al., 1997)
AP2 adaptin α-subunit	APA-2	(Yang et al., 2008)
AP1/2 adaptin β-subunit	APB-1	(Yang et al., 2008)
AP2 adaptin μ-subunit	DPY-23	(Pan et al., 2008a; Yang et al., 2008)
AP2 adaptin σ-subunit	APS-2	(Yang et al., 2008)
Retromer cargo-selective subcomplex component Vps26	VPS-26	(Coudreuse et al., 2006; Prasad and Clark, 2006)
Retromer cargo-selective subcomplex component Vps29	VPS-29	(Coudreuse et al., 2006; Prasad and Clark, 2006)
Retromer cargo-selective subcomplex component Vps35	VPS-35	(Coudreuse et al., 2006; Prasad and Clark, 2006)
Sorting nexin Snx3	SNX-3	(Harterink et al., 2011b)
Myotubularin lipid-phosphatase MTMR6	MTM-6	(Silhankova et al., 2010)
Myotubularin lipid-phosphatase MTMR9	MTM-9	(Silhankova et al., 2010)
Wnt	EGL-20	(Harris et al., 1996; Maloof et al., 1999)
Secreted Frizzled	SFRP-1	(Harterink et al., 2011a)
Frizzled	LIN-17	(Harris et al., 1996; Sawa et al., 1996)
	MIG-1	(Harris et al., 1996)
Dishevelled	MIG-5	(Walston et al., 2006)
GSK-3β	SGG-1	(Korswagen et al., 2002)
Axin	PRY-1	(Korswagen et al., 2002; Maloof et al., 1999)
	AXL-1	(Oosterveen et al., 2007)
β-catenin	BAR-1	(Korswagen et al., 2000; Maloof et al., 1999)
TCF/LEF-1	POP-1	(Herman, 2001; Korswagen et al., 2000)
Antennapedia	MAB-5	(Salser and Kenyon, 1992)

Another polarization event is e. g. the polarization of epidermal cell V5 among others by Wnt EGL-20 (Whangbo et al., 2000). I mention the EMS blastomere later so I paid more attention to it, but there are many other Wnt signalling events that are guided by different Wnts during the *Caenorhabditis elegans* development. They were mentioned in preceding chapter, but they are not the subject of this thesis and are thus not described in detail.

This thesis focuses on Wnt-dependent migration of the Q neuroblasts and their descendants. Wnt signalling guides the whole array of *C. elegans* future neurons migrations (Harris et al., 1996; Silhankova and Korswagen, 2007; Zinovyeva et al., 2008), polarizes cells, directs elongation of their growth cones (Hilliard and Bargmann, 2006; Prasad and Clark, 2006), determines the identity of the neuronal processes, their branching and the formation of synapses (Prasad and Clark, 2006). These events are usually operated by multiple Wnts and Frizzled receptors in a complex network which determine the precise location and morphology of the cell (Zinovyeva et al., 2008).

1.2.3 Migration of the Q neuroblasts

Migration of the QL neuroblast and its descendants represents an exception among other migrations of neurons, because it is guided by only one Wnt, EGL-20. One Q neuroblast is born in the same position on each side in the posterior half of the body, but each responds differently to EGL-20/Wnt. EGL-20 is produced by a group of epithelial and muscle cells surrounding the rectum posterior to the Q neuroblasts. Whereas the right Q neuroblast is not responsive to the Wnt signal and migrates to a default anterior direction, in the left Q neuroblast EGL-20/Wnt activates a canonical Wnt signalling cascade (Fig. 1). Activation of this pathway ultimately leads to stabilization of β-catenin BAR-1 (Korswagen et al., 2000) which together with POP-1/Tcf (Herman, 2001) activates transcription of target gene *mab-5*, an Antennapedia-like Hox gene cluster HOM-C homologue. QL and its descendants with activated *mab-5* then remain in the posterior (Salser and Kenyon, 1992).

In general, Q neuroblasts with *mab-5* ON remain in posterior, whereas Q neuroblasts with their *mab-5* gene turned OFF migrate to the anterior (Silhankova and Korswagen, 2007). Due to its relative simplicity (EGL-20 is the solely Wnt that governs the migration of Q neuroblasts) and a distinctive phenotype, migration of the Q neuroblasts represents a useful tool for studying Wnt signalling – when some

positive factor of the pathway, essential for its functioning, is eliminated, activation of *mab-5* in QL is prevented and both QL and QR migrate to the anterior. Because the QL neuroblast is then found in an abnormal position, this situation is referred to as „the QL phenotype“ and the experiment as the „QL assay“. In a rarer situation, when a negative regulator of the canonical Wnt signalling pathway in a target cell is mutated or knocked-down, the pathway is constitutively active in both QL and QR and both cells remain in the posterior, referring to as „the QR phenotype“.

The intrinsic assymetry of QL and QR in their response to EGL-20 signal is not the subject of this work and – as to my knowledge – has not been extensively studied, but netrin signalling has been suggested to establish this assymetry since when netrin receptor UNC-40 is missing, Q neuroblasts polarize randomly (Honigberg and Kenyon, 2000).

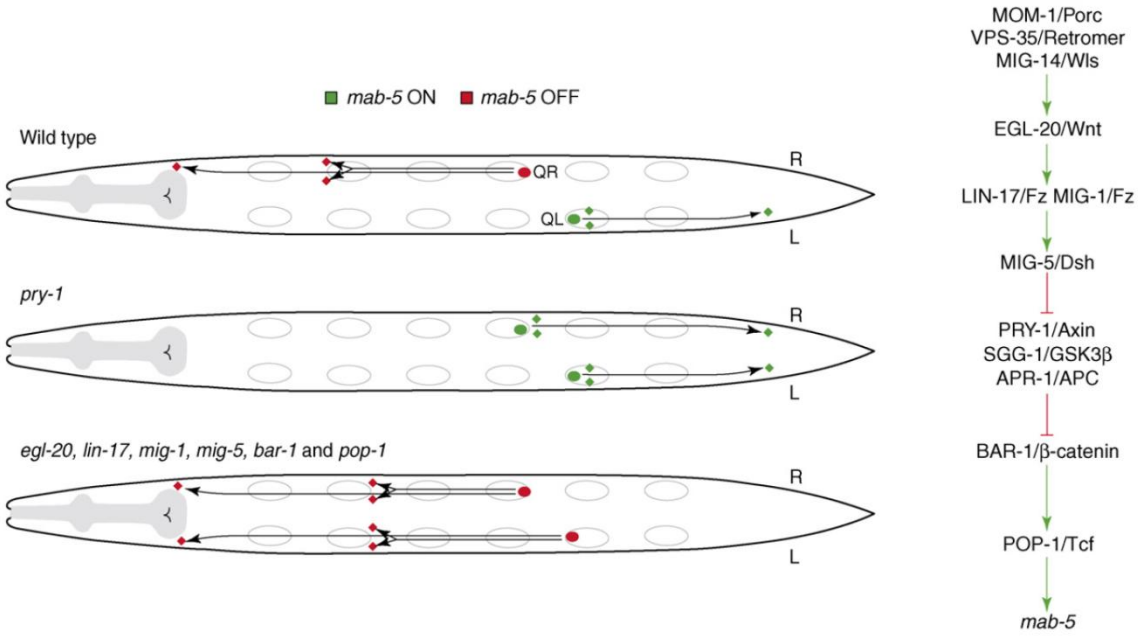

Figure 1: Migration of the Q neuroblasts

Schematic representation of migration of the Q neuroblasts and their descendants. Migration of the Q neuroblasts is guided by the Wnt signal produced in the tail. Binding of the Wnt ligand triggers the canonical Wnt signalling cascade in the QL but not the QR as described in more detail in the text. The whole signalling cascade from the production of a Wnt molecule to the activation of the target gene is shown in the right. Activating steps are in green, inhibiting ones are in red. Situation in wild type animals (top), mutants in negative (middle) and positive (bottom) regulators of Wnt signalling is shown. Dorsal view, anterior is left. Figure reprinted with permission from (Silhankova and Korswagen, 2007).

1.2.4 Making vs. reception of the Wnt signal

Since the situation downstream of the Wnt receptor Frizzled and its co-receptor LRP5/6 has been extensively studied and well described elswhere (e. g. (Kikuchi et al., 2009), see (Korswagen, 2002) for situation in *Caenorhabditis elegans*), I will focus in the following text on describing the less known events connected with Wnt synthesis, secretion and spreading. The attention has not been paid to this area until the last decade and although much work has been done in last years, new components and mechanisms are still being described. This thesis deals with one such potential new player in Wnt signalling and, as we expect, Wnt secretion, the protein CUP-4.

1.3 Secretion of the functional Wnt protein

This chapter covers the synthesis, secretion and spreading of Wnt proteins, i. e. the events preceding its binding to receptors of receiving cells. For most recent review of this topic see (Harterink and Korswagen, 2012). For easier orientation in the text, I also briefly summarize the process of Wnt production in the following section of this chapter.

1.3.1 Overview of the Wnt synthesis, properties, secretion and spreading

Wnts are hydrophobic acylated glycoproteins modified with many Cys-Cys bridges. Either glycosylations or acylations of Wnts require the action of the acyltransferase Porcupine in the endoplasmic reticulum. Wnt proteins are then transported bound to their cargo receptor Wntless (Wls) through the Golgi reticulum to the cell surface where they are released. Wls is then recycled for repeated use. Wnts spread most probably in the paracellular space with the aid of lipoprotein particles, heparan sulphate proteoglycans or bound to proteins from the lipocalin family. Alternatively, Wnts can be released on exosome-like vesicles together with their cargo receptors.

1.3.2 Posttranslational modifications of the Wnt proteins

1.3.2.1 Acylation

Wnts‘ posttranslational modifications were not known for a long time. After succesful purification of an active murine Wnt3A, it was found that Wnts are much more hydrophobic than expected from their amino acid sequences and display properties of membrane anchored proteins although they do not contain any long stretches of hydrophobic amino acids. It has been found, that Wnt3A is S-palmitoylated on Cys77 residue which is conserved among almost all Wnts studied, including *C. elegans* Wnt EGL-20 (Willert et al., 2003). Additionaly, Wnts are also modified by palmitoleic acid moiety on a conserved serine (Ser209 in murine Wnt3A; (Takada et al., 2006)).

Palmitoylation of Wnt3A C77 or a homologous cysteine in other Wnts is essential for Wnt hydrophobicity (Kurayoshi et al., 2007; Willert et al., 2003; Zhai et al., 2004) and it targets Wnt to lipid rafts (Zhai et al., 2004). While Wnt lacking palmitoleoylation of Ser is not secreted and is retained in the ER (Takada et al., 2006), mixed results were obtained with palmitoylation on Cys, which in some cases according to some authors is not required (Komekado et al., 2007; Kurayoshi et al., 2007), by others it is needed for secretion of Wnt (Zhai et al., 2004). Palmitic acid moiety on Cys is also essential for binding of Wnt to its Frizzled (Komekado et al., 2007; Kurayoshi et al., 2007; Zhai et al., 2004) and LRP6 receptors (Komekado et al., 2007). Overall, we can say, that acyl modifications of Wnts are essential for their activity and to some extent also for their secretion.

Candidate enzyme to perform acylations on Wnt proteins is porcupine, a Wnt processing protein encoded by a segment polarity gene from *Drosophila* (Kadowaki et al., 1996) or its *C. elegans* orthologue *mom-1*(Rocheleau et al., 1997). Porcupine is a putative member of membrane-bound O-acyl transferase superfamily (Hofmann, 2000) and resides in the ER of Wnt producing cells (Kadowaki et al., 1996). It is required for both S-palmitoylation and O-palmitoleoylation in Wnt producing cells (Takada et al., 2006; Zhai et al., 2004). Porcupine overexpression or presence enhances or stimulates palmitoylation, respectively (Galli et al., 2007; Komekado et al., 2007). Moreover, Porcupine co-immunoprecipitates with Wnt proteins (Tanaka et al., 2000).

Although actual modifications by acyl residues are not known for all Wnts, based on conservation of amino acids in modified sites, one can assume, that the modifications are also conserved. The only exception found so far is *Drosophila* WntD, which is not

lipid modified and does not require Porcupine or Wls/Evi/Sprinter (see section 1.3.4.1) for its secretion (Ching et al., 2008).

1.3.2.2 Glycosylation

Wnt proteins contain several potential N-glycosylation sites. Glycosylation is required for secretion (Komekado et al., 2007) but not for the activity of Wnt protein (Kurayoshi et al., 2007) and, like palmitoylation and paltmitoleoylation, depends on Porcupine. However, it is unlikely that Porcupine would be the enzyme that is directly responsible for glycosylation; Porcupine possibly through lipid modifications only brings Wnt molecules closer to the membranes, where they can be modified.

1.3.3 Wnt spreading

The means by which Wnt proteins spread in the tissues are tightly connected with their modifications and are hence discussed here, although chronologically they follow after the secretion of Wnt protein.

The Wnt proteins are too hydrophobic to easily spread by diffusion in aqueous phase. Multiple mechanisms of how their hydrophobicity could be overcome and spreading achieved were thus suggested – for review see e. g. (Port and Basler, 2010).

Wnt proteins were suggested to form micelles or to be transported by morphogen transporting vehicles called argosomes (Greco et al., 2001). The nature of such vehicles, however, remained unknown. (Panakova et al., 2005) found that argosomes are not the membraneous vesicles (exosomes) as initially thought but instead, *Drosophila* Wnt protein Wingless associates with lipoprotein particles produced in the fat body. Wnt must be first loaded on these extracellular vehicles. This probably requires endocytosis of lipoprotein particles or it is achieved by brushing of Wnt molecules from the cell surface where they concentrate.

But it was not until recently, when Swim, the *s*oluble *W*ingless *i*nteracting *m*olecule, was discovered (Mulligan et al., 2012). Swim belongs to the lipocalin family of proteins. Swim binds the palmitate residue of Wnt, thus concealing it from the aqueus environment. As a result, Swim ensures solubilization, spreading and long range activity of Wnt proteins (Mulligan et al., 2012).

Heparan sulphate proteoglycans (HSPGs) with Dally-like protein core bind Wnt proteins and pass them to the neighbouring cells thus enhancing long-range signalling

while Dally probabaly functions as a Frizzled co-receptor (Franch-Marro et al., 2005). Also Frizzled receptors themselves stabilize extracellular Wnt and shape its gradient (Baeg et al., 2004). Secreted Frizzled receptors cut off Wnt gradient at the anterior and thus help to spatially restrict the range of Wnt action (Harterink et al., 2011a).

1.3.4 Secretion of Wnt

1.3.4.1 The Wnt cargo receptor Wntless

C. elegans Wls homologue *mig-14/mom*-3 was described earlier to be essential in migration of Q neuroblasts (Harris et al., 1996; Sawa et al., 1996) and polarization of EMS blastomere by its neighbour P_2 (Rocheleau et al., 1997; Thorpe et al., 1997) but its nature remained unknown until 2006, when its function was independetly described by three groups. They „discovered“ it in screens for novel components of Wingless signalling in *Drosophila* and described as Wntless (Wls)/Evenness interrupted (Evi)/Sprinter (Banziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006) – from this place onwards referred to only as Wntless (Wls). Since this protein turned out to be the long-searched Wnt cargo receptor and it plays a central role in Wnt secretion, I will describe it to more detail.

Wls has eight predicted transmembrane domains, first of which overlaps partially with a signal sequence so there remains a possibility, that it is cleaved off during maturation. Wls physically interacts with Wnt (Banziger et al., 2006; Coombs et al., 2010) and is required for Wnt secretion – in Wls mutants, Wnt is not secreted and is instead retained in the producing cells (Banziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006), where it even does not reach the cell surface (Banziger et al., 2006). Wls was shown to be strictly specific for Wnt secretion as its mutation does not affect secretion of other proteins tested, most notably the secretion of Hedgehog, another lipid-modified morphogen with many characteristics similar to Wnt (Banziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006), or Udp, a ligand that activates JAK/STAT signalling in target cells (Bartscherer et al., 2006). Moreover, Wls mutants display no obvious phenotypes indicative of a disruption of signalling pathways other than Wnt (Banziger et al., 2006; Bartscherer et al., 2006) and can be rescued by expression of Wls from Wnt promoters (Banziger et al., 2006).

Interestingly, it has been found that Wls is not required for PCP-dependent orientation of bristles on notum of *Drosophila* adults (Bartscherer et al., 2006).

The authors explained this by a possibility that PCP in *Drosophila* might not be triggered by a Wnt ligand.

Conflicting results have been obtained for Wls localization – Wls-GFP fusion proteins localized to the plasma membrane (Bartscherer et al., 2006) while differently tagged Wls-HA was distributed mainly in the Golgi complex and small vesicles between the Golgi and the plasma membrane (Banziger et al., 2006). Later, Wls was observed to co-localize with the trans-Golgi network, endosomal and plasma membrane markers (Belenkaya et al., 2008; Franch-Marro et al., 2008; Port et al., 2008). Untagged (but not endogenous) Wls in human culture cells was localized primarily to the ER (Coombs et al., 2010).

The ER localization could be connected with Wls function – ER is the place, where Wnt modifications take place. While C77 palmitoylation or glycosylation are not required for binding of Wnt to Wls (Banziger et al., 2006; Komekado et al., 2007), it turned out that Wls by its β-barrell which is reminiscent of lipocalin fold (Wg transporting molecule Swim is also a lipocalin) binds to Ser209 (in murine Wnt3A) palmitoleate group. Vacuolar acidification (probably of exocytic vesicles) is necessary for Wnt to be released from Wls – if the acidification is inhibitited by V-ATPase inhibitors, Wnt-Wls complex cannot dissociate (Coombs et al., 2010).

One homologue of Wls/Evi/Sprinter was found in all analyzed metazoan genomes, but not in genomes of other organisms (Banziger et al., 2006). Until recently, Wls function was found to be required for action of all Wnts tested (Banziger et al., 2006; Bartscherer et al., 2006; Fu et al., 2009; Goodman et al., 2006; Rocheleau et al., 1997). Together, these data pointed to an ancient and universal but specific role of Wls in Wnt signalling. However, it was then discovered that XWntless is specifically required for secretion and activity of Xwnt4 but not other *Xenopus* Wnts (Kim et al., 2009).

The evidence that Wls is not involved only in secretion of Wnt but acts also in its transport and signal transduction in receiving cells comes from *Drosophila* larval neuromuscular junctions where Wls is released on exosome-like vesicles from pre-synaptic membranes (Korkut et al., 2009). Production of such Wls-containing vesicles is required for Wnt secretion suggesting that Wg could be transported by these vehicles together with Wls. In post-synaptic signal receiving cells, Wls targets protein dGRIP to the post-synaptic membranes. dGRIP in turn regulates trafficking of Wg receptor DFrizzled-2 (Korkut et al., 2009).

1.3.4.2 Role of the retromer

Retromer is a multiprotein complex which serves to retrieve cargo receptors of endosomal and lysosomal proteins back to the Golgi reticulum for repeated use as in case of Vps10p receptor of carboxypeptidase Y in yeasts (Seaman et al., 1997) and cation-independent mannose phosphate receptor in mammals (Arighi et al., 2004). It also promotes transcytosis of polymeric immunoglobulin A and its receptor (pIgA-pIgR) and regulates apical secretion of pIgA (Verges et al., 2004). The cargo-selective subcomplex of retromer is composed of VPS-35, VPS-29 and VPS-26 (Seaman et al., 1998); the second part of retromer which interacts with the endosomal membrane consists of homologs of sorting nexins (Horazdovsky et al., 1997; Seaman et al., 1998).

Retromer function was reported to be important for several Wnt dependent processes in *C. elegans* like migration of the Q neuroblasts guided by Wnt EGL-20 (Coudreuse et al., 2006) and for neuronal polarity, axonal growth and branching guided by multiple Wnts (Prasad and Clark, 2006). The importance of retromer for Wnt signalling was confirmed on mammalian cells and *Xenopus* embryos (Coudreuse et al., 2006). Together, these results point to a universal and evolutionary conserved function of retromer in Wnt signalling. Retromer was shown to be specifically required in Wnt producing cells (Coudreuse et al., 2006; Prasad and Clark, 2006). Staining of EGL-20/Wnt revealed that the Wnt concentration gradient outside Wnt producing cells was greatly reduced or absent in retromer mutants (Coudreuse et al., 2006). Long-range events, like migration of QL neuroblasts, which respond to the signal from a distance of several cell diameters, were observed to be more affected then short-range events (like polarization of the V5 cell). Initially, no reduction in secretion of Wnt was detected in retromer-depleated cells. It was thus proposed, that retromer is essential for production of a specific pool of Wnt capable of diffusion over distance, for example Wnt associated with lipoprotein particles (Coudreuse et al., 2006). Later the role of retromer in Wnt producing cells was better understood (see next section of this chapter) - other experiments showed that secretion of Wnt in retromer mutants is in fact compromised and that activation of downstream target genes and processes depends probably more on their thresholds, i. e. whether the remaining Wnt level is sufficient for their activation. This is why the original hypothesis was largely abandoned in favor of the idea, that lower amount of secreted Wnt protein forms a shallower concentration gradient (Pan et al., 2008a; Yang et al., 2008).

Surprisingly, *C. elegans vps-29* mutants have fewer and less severe Wnt-related defects than either *vps-26* or *vps-35* mutants, so it seems, that VPS-29 is not so essential for Wnt signalling in *C. elegans* as the other two components of the cargo-selective retromer subcomplex (Prasad and Clark, 2006).

1.3.4.3 Retrograde transport of Wntless

Despite the importance of retromer for Wnt signalling was clearly demonstrated, the protein or process regulated by retromer still remained unknown. The findings that both Wls and retromer are required in Wnt producing cells and both display similar mutant phenotypes (impaired secretion of Wnt protein) suggested that Wntless could be this mysterious molecule. And indeed, five research groups in the same year simultaneously discovered, that retromer ensures the recycling of Wls from early endosomes to the trans-Golgi network in *Drosophila*, *Caenorhabditis* and cultured mammalian cells (Belenkaya et al., 2008; Franch-Marro et al., 2008; Pan et al., 2008a; Port et al., 2008; Yang et al., 2008), later confirmed also on Xenopus (Kim et al., 2009).

Wntless was found to physically interact with retromer: these proteins can co-immunoprecipitate (Belenkaya et al., 2008; Franch-Marro et al., 2008) and they also colocalize on endosomes (Belenkaya et al., 2008; Franch-Marro et al., 2008; Yang et al., 2008). In retromer mutants, the levels of Wntless are reduced probably because it is not recycled to TGN but instead is subjected to increased degradation in lysosomes (Yang et al., 2008). The secretion of Wnt protein is thus largely prevented so much lower amount of extracellular Wg is detected around Wg producing cells of *Drosophila* wing discs, where it is normally present as a punctate staining. Instead, Wnt is retained and accumulates in the secreting cells (Belenkaya et al., 2008; Franch-Marro et al., 2008; Port et al., 2008). The expression of Wnt protein is not altered at a transcriptional or protein level (Belenkaya et al., 2008; Pan et al., 2008a; Port et al., 2008), so it seems that it cannot be transported out of the producing cell. Overall, these observations were explained by the idea that retromer is required for Wls to be recycled back from endosomes to the trans-Golgi network for next round of Wnt secretion. When retromer function is missing, Wls is instead degraded in lysosomes. Consistent with this model, overexpression of Wls was found to be sufficient to rescue Wnt defects of retromer mutants (Belenkaya et al., 2008; Franch-Marro et al., 2008; Pan et al., 2008a; Port et al., 2008; Yang et al., 2008).

The retromer complex was also found to be required for action of *Xenopus* XWnt1, XWnt3, XWnt4, XWnt5, XWnt8 and XWnt11 but only in XWnt4 it functions in Wls-dependent manner to ensure the secretion of Wnt protein. In other Wnts retromer, in contrast, acts in a Wls-independent manner and does not affect secretion. Instead, it may affect production of a long range acting form of Wnt or it plays a role in signal receiving cells (Kim et al., 2009).

Since Wls is transported back to the TGN from endosomes, it clearly has to first get there – this is achieved probably through clathrin-mediated endocytosis. Because the loss of clathrin function is lethal, it was not tested for Wnt signalling defects itself, but it was demonstrated that AP2, a heterotetrameric adaptin complex involved in clathrin mediated endocytosis, is required in Wnt producing cells for functional Wnt signalling. Mutation or knockdown of AP2 μ subunit gene *dpy-23* or the knock down of the other AP2 subunits (*apa-2*, *aps-2* and *apb-1*) in *C. elegnas* result in Wnt-related phenotypes including EGL-20/Wnt dependent migration of the Q neuroblasts (Pan et al., 2008a; Yang et al., 2008). AP2 ensures the internalization of MIG-14/Wls from the plasma membrane since when AP2 is knocked down, MIG-14::GFP accumulates on the plasma membrane of the Wnt producing cells where it is homogenously distributed (Yang et al., 2008). The levels of MIG-14::GFP are higer than normally in both wild type and retromer mutants, because MIG-14 is protected from internalization and degradation in lysosomes.

Another completely different layer of Wls regulation is represented by the control of its transcription – e. g. mouse Wls ortologue Gpr177 was found to be a target gene of Wnt signalling and its transcription is activated in a positive feedback loop by Wnt3 (Fu et al., 2009). This is slightly in contrast to findings made by (Port et al., 2008) who also found elevated expression of Wntless in Wnt producing cells but this expression was not caused by increased transcription due to activation of Wnt signalling. Instead, in seemed to be set and stabilized by the simple presence of Wnt protein alone.

1.3.4.4 Sorting nexin SNX-3

SNX-BAR sorting nexins recruit the retromer cargo-selective subcomplex to endosomal membranes through binding to phosphatidylinositol-3-phosphate or PI-3,5-P_2. They induce membrane deformation with their BAR domains leading to formation of a tubule (Carlton et al., 2004). These canonical sorting nexins were, however, found

not to function in retrieving the Wnt cargo receptor Wntless from endosomes back to TGN (Harterink et al., 2011b).

Instead, a Phox-homology (PX) domain-only sorting nexin SNX-3 was shown to be essential for Wnt signalling in *Drosophila*, *C. elegans* and cultured mammalian cells (Harterink et al., 2011b). SNX-3 is required in EGL-20/Wnt producing cells where it regulates stability of MIG-14/Wntless and colocalizes with Wntless, the retromer cargo-selective subcomplex (Harterink et al., 2011b) and PI-3-P (Silhankova et al., 2010) on vesicles budding from endosomes.

According to the proposed model, SNX-3 binds with its PX domain 3-phosphoinositides and recruits retromer cargo-selective subcomplex and Wntless to PI-3 containing endosomal membranes (Harterink et al., 2011b; Silhankova et al., 2010). Resulting vesicles are morphologically distinct from the tubules present in canonical SNX-BAR pathway (Carlton et al., 2004). Since SNX-3 lacks BAR domain which force the membrane to deform, clathrin was suggested to perform this role in SNX-3-retromer-dependent Wls trafficking (Harterink et al., 2011b).

1.3.4.5 Myotubularin phosphoinositide 3-phosphatases

Since SNX-3, the retromer recruiting protein, is itself brought to membranes by binding to 3-phosphoinositides, proteins regulating proper levels and balance of these phosphorylated forms of lipids are also very likely to regulate binding of retromer and hence Wntless recycling and Wnt signalling. Indeed, myotubularin PI 3-phosphatases were found to be essential for Wnt signalling (Silhankova et al., 2010).

Myotubularins represent a family of proteins comprised of myotubularin MTM1 and myotubularin-related proteins MTMR2-13. MTM1 was found to be the cause of myotubular myopathy, congenital disease affecting muscles, and MTMR2 of Charcot-Marie-Tooth disease, a neural degenerative disorder (reviewed in (Laporte et al., 2003)). Myotubularins were discovered to be enzymatically active or inactive lipid phosphatases which dephosphorylate phosphatidylinositol phosphates on their third position (Taylor et al., 2000).

In *C. elegans*, myotubularins were first observed in a screen for Cup mutants as genes, whose functions are necessary for efficient endocytosis by coelomocytes, specialized phagocytic cells in the body cavity – hence the name „Cup“ which comes from „*c*oelomocyte *up*take“. Later, *cup-6* was homologized with mammalian MTMR6

and renamed to *mtm-6* while *cup-10* was homologized to MTMR9 and renamed to *mtm-9* (Dang et al., 2004). These two myotubularins are components of the same complex, probably a heterodimer, where *mtm-6* represents the catalytically active subunit and *mtm-9* its catalytically inactive binding partner which regulates *mtm-6* activity (Dang et al., 2004). Overall, *C. elegans* has six myotubularins and majority of them functions in coelomocyte endocytosis, where they probably regulate levels of specific PI-3-P pools (Xue et al., 2003) in a pathway that encompasses small GTPase ARF-6 and RME-1 (Dang et al., 2004) .

As clathrin-dependent endocytosis is also essential for efficient recycling of Wnt cargo receptor Wntless as well as budding of vesicles from early endosomes, where PI-3-P is most abundant, all Cup mutants were screened for defects in EGL-20-dependent migration of Q neuroblasts. Among them, the only genes with strong effect were *mtm-6* and *mtm-9* (Silhankova et al., 2010).

MTM-6 (and hence also its binding partner MTM-9) are required specifically in EGL-20/Wnt producing cells for Wnt signalling since mutant phenotype of *mtm-6* mutants can be rescued by expressing *mtm-6* from an *egl-20* promoter. Interestingly, *mtm-6* phenotype is not as severe as that of *mtm-9*, probably because of other myotubularins can partially substitute for MTM-6. At least, this is suggested by enhancement of the QL migration defect by *mtm-5* to *mtm-9* level in *mtm-6; mtm-5* double mutant and by *mtm-1* or *mtm-3* RNAi on *mtm-6* mutant background (Silhankova et al., 2010). Defect in Wnt signalling in *mtm-6* mutant animals is caused by an impaired recycling of MIG-14/Wls. MIG-14/Wls in EGL-20/Wnt producing cells of *mtm-6* mutants is instead targeted for degradation in lysosomes and its levels are reduced (Silhankova et al., 2010), a condition, under which Wls becomes limiting for Wnt signalling. As a result, mainly long-distance signalling events, like the QL neuroblast migration, are affected, a phenotype that strongly resembles retromer mutants (Coudreuse et al., 2006).

In EGL-20/Wnt producing cells, myotubularins also function through modulation of PI-3-P levels, since knockdown of gene *vps-34* encoding PI-3 kinase restores EGL-20 signalling.(Silhankova et al., 2010). Phosphatidylinositol-3-phosphate subsequently recruits sorting nexin SNX-3, a component of retromer, to Wls containing vesicles budding from early endosomes (Harterink et al., 2011b; Silhankova et al., 2010).

1.3.5 Current model of the Wnt secretion and spreading

After its synthesis into the lumen of ER, cysteine bridges are formed and Wnt proteins are palmitoylated, palmitoleoylated and glycosylated dependent on the activity of the O-acyltransferase Porcupine. Wnts then pass through the Golgi apparatus, where they bind to their cargo receptor Wntless which transports them to the plasma membrane. Wnts are released and bind to Swim, a soluble Wnt-transporting molecule. They can be also loaded on HPSGs and passed from cell to cell, endocytosed and attached to the extracellular vehicles called argosomes and represented by lipoprotein particles or transported on exocytic vesicles together with Wntless. However, in a normal situation, Wls is recycled from the cell surface back to the trans-Golgi reticulum for repeated use so it can participate on the next round Wnt secretion (Figure 2). Wls is internalized through binding to adaptin AP2 and clathrin mediated endocytosis. In early endosomes, Wls concentrates in the membranes with elevated PI-3-P levels which is synthesized by VPS-34 kinase. PI-3-P recruits SNX-3 and cargo-selective subunit of the retromer composed of VPS-26, VPS-29 and VPS-35 resulting in pinching off the vesicles which recycle Wls back to the trans-Golgi network so it can be used in the next round of the Wnt secretion. This recycling of Wls is essential to maintain Wls levels and to ensure sufficient production of the Wnt protein. When this loop of Wls recycling is disrupted, Wls is instead diverted to lysosomes and degraded. Under these conditions, Wls becomes limiting for the secretion of the Wnt protein and the whole Wnt signalling becomes compromised.

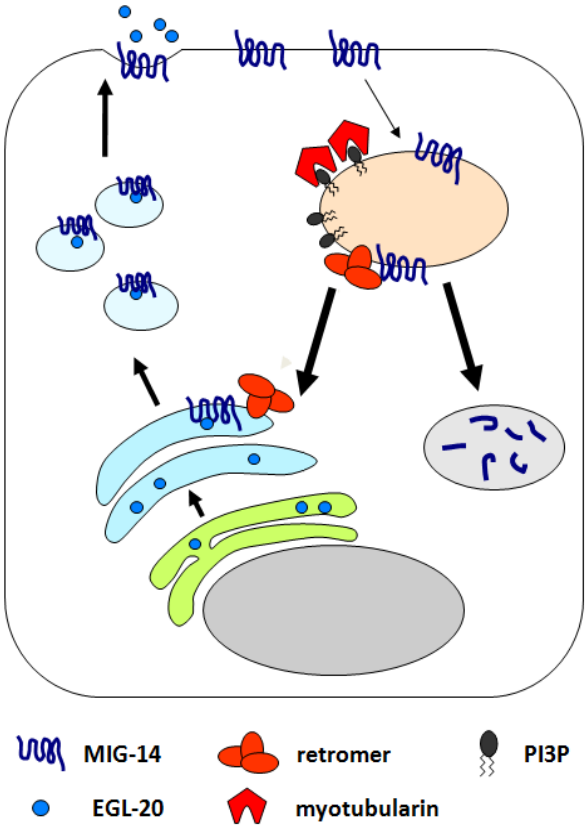

Figure 2:

The model of Wls recycling in Wnt secreting cells

For description see the text (figure courtesy of Mgr. M. Macůrková, Ph.D., unpublished)

1.4 *cup-4*

When *C. elegans* mutants defective in endocytosis were examined for an effect on Wnt signalling, a *cup-4* mutant was shown to have a mild effect on EGL-20/Wnt dependent migration of the QL.d neuroblasts (Silhankova et al., 2010). Although the effect was not so prominent as in *mtm-6, mtm-9* or other known components of Wnt signalling like MIG-14/Wls or retromer complex subunits (Banziger et al., 2006; Bartscherer et al., 2006; Coudreuse et al., 2006; Goodman et al., 2006; Harterink et al., 2011b), it was statistically significant (Silhankova et al., 2010).

CUP-4 is a 433 amino acid membrane protein (Patton et al., 2005) and the member of a ligand-gated ion channel receptor family (Jones and Sattelle, 2004). It shows the highest degree of homology with nicotinic acetylcholine receptors (Patton et al., 2005) and may resemble GABA or glycin receptors (Jones and Sattelle, 2004). Similar to nAChRs, it has four predicted α-helical transmembrane domains at its C-terminus and the hallmark of ligand-gated ion channels, 13 amino acids flanked by two cysteine residues (the „Cys-loop“ motif) in its extracellular domain (Patton et al., 2005). Unlike nAChRs ligand binding alpha-subunits, CUP-4 has no known ligand, so it belongs to a group of so called non-α orphan receptors (Jones and Sattelle, 2004).

1.4.1 CUP-4 function in endocytosis

As suggested by its name (*cup* stands for *c*oelomocyte *up*take defective), *cup-4* mutant was first isolated in a mutagenic screen searching for defects in endocytosis by coelomocytes, specialized phagocytic cells that nonspecifically endocytose fluid from body cavity (Fares and Greenwald, 2001). In wild type animals, coelomocytes endocytose and concentrate most of the GFP secreted into the pseudocoelom from body wall muscle cells of transgenic animals, whereas coelomocytes of *cup-4* mutants do not. Although it still was not conclusively proven, it is very likely that the majority of endocytosis by coelomocytes is clathrin-dependent (Fares and Greenwald, 2001) similar to endocytosis of Wls in Wnt producing cells (Pan et al., 2008a; Yang et al., 2008).

cup-4 is specifically expressed in coelomocytes and CUP-4::GFP fusion protein expressed under a coelomocyte-specific promoter was observed to localize to the Golgi apparatus and/or to the plasma membrane/endosomes (Patton et al., 2005).

Apart from the defective endocytosis, *cup-4* mutants have drastically reduced levels of phosphatidylinositol-4,5-bisphosphate ($PI_{4,5}P_2$) in their cytoplasmic membranes (Patton et al., 2005). In addition, *cup-4* mutants display some mild defects common to all mutations disturbing coelomocyte function (Patton et al., 2005). For example, lysosomes are smaller in size than they are in wild type animals, but this, as well as scattered endoplasmic reticulum in *cup-4* mutants, seems to be just an effect of decreased intake of fluids and membrane. *cup-4* mutation blocks endocytosis prior to the entry of endocytosed material to early endosomes, although some material can pass to vesicles with lysosomal markers (Patton et al., 2005). When genetic interactions were tested, CUP-4 was found to function downstream of or in parallel with dynamin DYN-1 and calcineurin A/B TAX-6/CNB-1 (Song et al., 2010).

1.4.2 CUP-4 in oxidative stress response and dietary resctriction induced longevity

It has also been shown, that *cup-4* expression is upregulated in response to oxidative stress and that its knockdown reduces oxidative-stress resistance (Park et al., 2009). Induction of *cup-4* expression under oxidative stress (posed by hyperbaric oxygen or diverse chemicals producing free radicals) is mediated by transcription factor SKN-1, which is acting upstream of CUP-4, since knockdown of SKN-1 largely prevents *cup-4* overexpression in these conditions (Park et al., 2009).

Both mean and maximum life span were observed to be shorter in *cup-4 (ok837)* mutants (Park et al., 2010) or worms exposed to *cup*-4 RNAi (Park et al., 2009) and *cup-4* RNAi reduced extended longevity induced by dietary restriction (DR) but not longevity of other long-lived mutants (Park et al., 2010). The same reduction in DR mediated longevity was also observed in *lgc*-26 RNAi, other coelomocyte-endocytosis-specific gene *cup*-5 and general endocytic genes (Park et al., 2010). In all these processes, CUP-4 appear to function together in the same pathway with NLP-7, a neuropeptide-like protein expressed in neurons and involved in their signalling (Nathoo et al., 2001). RNAi of one of these genes on the mutated background of the other causes no additional effect on life span. One might expect, that CUP-4 could be the receptor of NLP-7, but NLP-7 is predicted to bind to cholecystokinin receptors (Park et al., 2010). No induction of *nlp-7* or *pha-4*, the transcription factor specific for DR mediated life span extension, was observed in *cup-4 (ok837)* raising the possibility, that CUP-4 acts upstream of these two

proteins (Park et al., 2010). Overexpression of *cup-4* from a heat-shock promoter interestingly also leads to a shorter life span and, moreover, it causes decreased fertility and delay in egg-laying (Park et al., 2010).

1.4.3 *cup-4* paralogue *lgc-26*

There is another nAChR superfamily member, that exhibit the same „Cup" phenotype, *lgc-26*. Its knockdown on *cup-4* mutant background causes no additional defects and *vice versa* (Patton et al., 2005). This suggests, that LGC-26 acts in the same process as CUP-4. These two proteins could even possibly be the different subunits of the same oligomeric nAChR family ion channel. Then, the endocytic and probably also Wnt signalling defect could be the result of loss of channel function. On the other hand, roles of both proteins in dietary restriction response (see below) support the idea of CUP-4 acting upstream of LGC-26 (Park et al., 2010).

2 Objectives

In order to get a better understanding of CUP-4 role in Wnt signalling, we set ourselves the following objectives:

1) Determine CUP-4 tissue specificity in Wnt signallling, i. e. whether it is required in Wnt producing or receiving cell, and if possible, also the site of its action in Wnt signalling pathway.

2) Explore genetic interactions between *cup-4* and other genes whose products are involved in Wnt signalling or could mediate CUP-4 function.

3) Try to propose and test model(s) of mechanism of CUP-4 function in Wnt signalling.

3 Hypotheses

3.1 Place of CUP-4 action in Wnt signalling

When thinking about the place of action of CUP-4 in Wnt signalling, there are basically three possibilities to deal with:

1) CUP-4 acts in the Wnt producing cells.
2) CUP-4 plays a role in the signal receiving cell
3) CUP-4 affect Wnt signalling indirectly, for example by its function in coelomocyte endocytosis

3.2 Mechanisms of CUP-4 function

There are several ways to explain all known *cup-4* mutant phenotypes, i. e. how could CUP-4 simultaneously affect endocytosis, phosphoinositide levels and Wnt signalling. No matter where CUP-4 function takes place in the Wnt cascade, in all of the three cases mentioned above, the defect in Wnt signalling could result from defect in endocytosis observed in *cup-4* mutants. This could be achieved in following ways:

ad 1) In Wnt producing cells, endocytosis is essential for recycling of Wnt cargo receptor Wntless for repeated use necessary for secretion of sufficient amount of Wnt.

ad 2) In Wnt receiving cells, endocytosis could be required for internalization of Wnt ligand – Frizzled-LRP5/6 receptor complexes and sustained signalling

ad 3) In other cells of the body, endocytosis could play a role in transcytosis of Wnt protein (possibly bound to some kind of extracellular vehicles) on its way from source to the target tissue.

PI-4,5-P_2 is a regulator of endocytosis (Di Paolo and De Camilli, 2006) necessary for the binding of AP2 clathrin-mediated endocytosis adaptin complex (Höning et al., 2005). It is therefore tempting tu speculate, that all the *cup-4* phenotypes could be explained by the disruption of a single CUP-4 function which leads to the diminished levels of PI-4,5-P_2. Following possibilities of how this is achieved can be envisaged:

i) modification of PI phosphatase activity.
ii) modification of PI kinase activity.

iii) modification of phospholipase C activity

All the proteins mentioned could be regulated directly or indirectly (for example through ARF-6 and/or PI 5-kinase)

Alternatively, the lack of functional CUP-4 protein could lead (through the elimination of its ion channel activity or the activation of phospholipase C and subsequent release of inositol-1,4,5-trisphosphate) to an increase in the concentration of Ca^{2+}. Calcium ions would then activate calcineurin phosphatase. Calcineurin, in turn, would dephosphorylate and inactivate dynamin, thus preventing endocytosis (Song et al., 2010). However, only the scenario with hyperactivated phospholipase C, accounts with shifted balance in phosphoinositide levels in *cup-4* mutants.

On the other hand, the elimination of CUP-4 function can cause Wnt signalling defect in parallel to its endocytic defect and the reduced levels of PI-4,5-P2 in *cup-4* mutants could be achieved through a different mechanism or they could be simply just a by product of elimination of the process which also leads to the Wnt signalling defect.

When we temporarily accept the hypothesis that CUP-4 functions in Wnt signal producing cells, there are several options of how CUP-4 could function in these cells:

I) CUP-4 plays a role in EGL-20/Wnt synthesis, folding or modifications.

II) *cup-4* mutation prevents Wnt (and probably also its cargo receptor Wntless) from reaching the plasmatic membrane.

III) CUP-4 is required for the endocytosis of Wls from the cytoplasmic membrane to endosomes, probably by maintaining PI-4,5-P_2 levels. PI-4,5-P_2 recruits AP2, whose binding is necessary for the internalization of Wls (Pan et al., 2008a; Yang et al., 2008), to the cytoplasmic side of the membrane and enables the binding of the cargo proteins (Höning et al., 2005).

IV) CUP-4 is essential for efficient retrieving of Wntless from endosomes to the TGN for repeated use. Based on *cup-4* PI-4,5-P_2 phenotype, CUP-4 could affect Wntless recycling by also modulating the levels of 3-phosphoinositides. This could be achieved through:

i) Modification of PI 3-phosphatase activity.

ii) Modification of PI 3-kinase activity.

iii) Modification of phospholipase C activity.

4 Materials and methods

4.1 *C. elegans* and bacteria strains

4.1.1 *C. elegans* strains

Bristol N2 was used as a wild type strain and constitutes background for all mutations or transgenes used. Strain carrying *muIs32* transgene on wild type background was used as a control for RNAi and from now on it will be referred to as CF700 (the strain's code).

4.1.1.1 Mutations

Table 2 **Mutant alleles**

Linkage group (LG)	Mutant allele	Reference
I	*pry-1 (mu38)*	(Maloof et al., 1999)
II	-	-
III	*cup-4 (ok837)*	(Fares and Greenwald, 2001)
	mtm-6 (ok330)	(Silhankova et al., 2010)
	vps-29 (tm1320)	

4.1.1.2 Transgenes

Table 3 **Transgenes**

Linkage group (LG)	Transgene	Reference
I	*arIs37[Pmyo-3::ssgfp; dpy-20 (+)]*	(Fares and Greenwald, 2001)
II	*muIs32[Pmec-7::gfp; lin-15(+)]*	(Ch'ng et al., 2003)
	huSi2[Pmig-14::mig-14::gfp]	(Silhankova et al., 2010)

4.1.2 Bacteria strains

Following strains of *Escherichia coli* were used:

OP50	used to feed *C. elegans* during common cultivation
HT115	used for *C. elegans* feeding RNAi protocol
TOP10	competent cells used for cloning
DH-5α	competent cells used for cloning

4.2 Worms culturing and manipulation

Worms were cultured on agar plates with grown lawn of *Escherichia coli* OP50 strain. For 1 liter of agar, I used 3 g of NaCl, 2,5 g of tryptone (BactoTryptone, BD Biosciences) and 17 grams of BactoAgar (BD Biosciences). After adding water up to one liter, agar was autoclaved in 120 °C, cooled down to approximately 60 – 70 °C and then 25 ml of K-phosphate buffer (pH = 6.0), 1 ml 1M $CaCl_2$, 1 ml 1M $MgSO_4$ and 1 ml cholesterol (5 mg/ml in EtOH) were added. The resulting mixture was then pipetted into plastic Petri dishes with diameters of 60 milimeters for common culturing, 30 mm for crossings and 90 mm when greater amounts of worms were needed (e. g. preparation of samples for Western blot or freezing of newly prepared strains). Plates were left on the table overnight to let the agar dry. Simultaneously *E. coli* OP50 was inoculated from a colony on an agar plate into appropriate amount of LB medium in a glass tube and cultured overnight with shaking. Next day, 50 μl (for 3 and 6 cm plates) or 100 μl (for 90 mm plates) of *E. coli* LB culture was pipetted onto plates. In addition, bacteria on 60 and 90 mm plates were spread over the entire surface with a sterile glass stick. The plates with bacteria were left on the table overnight to grow a lawn and then stored upside down in plastic boxes in the fridge up to several weeks for continuous use.

Worms were transferred to new plates by dissecting roughly 0,25 cm^2 piece of agar (a „chunk“) from the old plate with a scalpel and transferring it upside down onto a new plate. Alternatively, worms were transferred one by one with a platinum wire attached to glass stick. The latter method was used especially for crossings and maintaining unstable transgenic strains when specific individual worms needed to be transferred, while the first one was the common method of maintaining stable strains. Scalpel was dipped into denatured ethanol and (as well as the platinum wire) schorched in the gas burner before/after each use to prevent contamination. Plates with worms were

sealed with Parafilm and stored upside down in plastic boxes or freely in thermostats at 20 or 15 °C (set temperature, actual value was actually more often around 16 ± 1 °C) or, when needed, on the table in the laboratory temperature.

When needed, worms were washed from plates with M9 buffer (22mM Na_2HPO_4, 22mM KH_2PO_4, 85mM NaCl, 1mM $MgSO_4$; 800 µl per 6 cm plate, 2 × 800 µl per 90 mm plate) to 1,5 ml Eppendorf microtube and pelleted down in benchtop microcentrifuge at 4000 rpm for 30 seconds.

4.2.1 Synchronization

Some experimented demanded using of synchronized worm populations, i. e. all or most of the worms in the population should be of the same defined stage. Two methods to obtain synchronized populations of *C. elegans* were used, hypochlorite treatment and washing.

4.2.1.1 Hypochlorite treatment

Worms from a fully grown plate (ideally with many adults filled with unlaid embryos) were washed with M9 buffer to 1,5 ml Eppendorf microtube and pelleted down in benchtop microcentrifuge (30 sec., 4000 rpm). Most of the supernatant was discarded and only approximately 50 µl with pelleted worms were left. Fresh M9 buffer was added to the worms to increase volume up to 700 µl and subsequently, 300 µl of mixture of hypochlorite solution (SAVO) : 5M KOH in 2:1 ratio was added. The mixture was then incubated in laboratory temperature for 5-6 minutes or until the worms body wall broke down and unlaid embryos were released. After that, embryos were immediately pelleted by centrifugation, supernatant discarded and pellet washed three times with 1 ml of M9 buffer. Every time, after adding of buffer, microtubes were centrifuged and supernatant discarded before adding the new buffer. Embryos in the pellet were used to obtain synchronized population of worms. This protocol was also used to get rid of yeast and bacterial contaminations from strain stocks.

4.2.1.2 Washing

In cases like confocal fluorescent microscopy, I needed animals only few hours old,. For these purposes, I used a simpler protocol of synchronization by washing:

The plate with many laid embryos was washed 1-2 times with M9 buffer, so only laid embryos that sticked to the surface remained. The plate was re-sealed and cultivated for few hours to let the larvae to hatch.

The advantages of this method over hypochlorite treatment are obvious: although there always remain few older animals that were not succesfuly washed away, the population of worms obtained by washing is better synchonized. Washing also does not pose so much stress on the worms as the hypochlorite treatment and hence is more physiological. As I suggest in this work, various forms of stress can enhance increase the penetrance of some mutant phenotypes (see chapter Results, section 5.1 QL migration defect of *cup-4* mutants is sensitive to cultivation temperature) and thus affect results of the experiments.

4.3 Crossings

To introduce a mutation or transgene into another mutant background in order to generate double mutants or strain carrying transgene on mutant background, two strains, each carrying one transgene or mutation, had to be crossed.

For crossings, it is important to know the mechanism of sex determination. In *C. elegans*, animals with the set of two X chromosomes (genotype XX) are hermaphrodites, while individuals with only one X chromosome develop into males. Hermaphrodites can reproduce on their own by self-fertilizing, so when homozygotic in some gene, their progeny is again homozygotic, while when parent hermaphrodite is heterozygotic in some gene, its progeny assorts in agreement with Mendel‘s laws. When hermaphrodite homozygous for an allele of a certain gene is fertilized by a male homozygous for a different allele of the same gene, all their progeny is heterozygous in this gene, although in the progeny of the hermaphrodite a various number of individuals originated by self-fertilizing can occur.

4.3.1 General crossing strategy

First, males carrying mutant allele or transgene had to be generated. For this purpose, usually 8-12 N2 wild type strain males (ideally L4 or young adults) were transferred to 3 cm agar plate along with 3-4 L4 hermaphrodites of strain mutated in gene A. After few days of cultivation, F1 males (heterozygotic in the gene of interest) were picked from the progeny and crossed in the same way with L4 hermaphrodites

of the strain, which carry mutation/transgene B. From the resulting F2 progeny, usually 8 to 10 animals were picked and separated onto new 3 cm plates. They should be all heterozygotic in mutation/ transgene B (except for progeny originated by self-fertilization), so after laying some embryos and hatching of enough larvae, F2 generation adult parent hermaphrodite was picked from each plate and genotyped for the mutation A (for genotyping strategy see the next section of this chapter). From F3 progeny of F2 heterozygotes, overall around 30-32 animals were picked and transferred each onto a new separate plate. After laying enough embryos and hatching of larvae, F3 parents were picked and genotyped for both mutations and/or examined for presence of the transgene. If double mutant homozygote was not found, F4 progeny of F3 mutant homozygote in one gene and heterozygote in the other was picked for another round. Among them, double mutant homozygotes were found by genotyping and their F5 progeny was used to establish a new double mutant or mutant/transgenic strain line.

4.3.2 Genotyping

To assess the genotype (with respect to one or two gene(s) of interest), I collected one to several worms into 0,5 ml microtube or 0,2 ml microtube strip with 10 µ of lysis buffer (30 mM Tris pH 8, 8mM EDTA, 100mM NaCl, 0,7% NP40, 0,7% Tween 20) with proteinase K (100µM). Worms were lysed in PCR machine set to 1 hour for 65 °C followed by 15 minutes of 95 °C to inactivate the proteinase. Protein lysates were used immediately and/or stored in the freezer in –20 °C for later use as templates for genotyping. From worm lysates, deletion alleles could be detected by PCR with two sets of primers, where one primer was common to both sets and was located somewhere in the non-deleted region of the gene, either in its coding or non-coding region.

The second primer of first set is located elswhere in the non-deleted region of the gene so the deleted region is located between forward and reverse primers. From mutated allele the PCR then yields a shorter product then from the wild type allele. This set of primers hence serves to detect the presence of the mutant allele.

The second primer of the second set is complement to the deleted region, so PCR product is made only when wild type allele is present. This is why this set of primers allows to detect the presence of the wild type allele.

Together, these two sets of primers allow to distinguist between heterozygotes, wt or mutant homozygotes.

PCR reactions (20 μl) were mixed as followed:

Master mix for all reactions with same primers was prepared by multiplying following amounts by number of samples approximated up by adding at least 2-3 to have some reserve. 19 μl of master mix was pipetted into each 0,2 microtube/microtube stripe/well of 96 well-plate before adding of template.

For 1 PCR reaction was used:

15,25 μl	deionized water
2 μl	DreamTaq buffer (Fermentas)
0,5 μl	dNTPs (10μM)
0,5 μl	primer forward (10μM)
0,5 μl	primer reverse (10μM)
0,25 μl	Taq polymerase in glycerol (own production)

1 μl of worm lysate was added as a template into each reaction.

Typical setting of PCR for genotyping was:

94-95 °C	2 minutes	initial denaturation
---		
cycle (35×):		
94-95 °C	20 seconds	denaturation
56-58 °C	40 seconds	annealing
72 °C	40 sec.-1 min.	synthesis
---		
72 °C	5 minutes	final synthesis
12 °C	-	hold

Following sets of primers were used for genotyping of individual genes:

cup-4

Forward:	CAGACAATGACAGTTCATGGAC
Reverse 1:	AAAGTGCTGAGCTTGAGACG
Reverse 2:	TACGCAAATCGGGAACGCAC

mtm-6

Forward 1: ATGTGCACAGCCTCTGACG

Forward 2: TCTCATCGAGAAGGATTGGC

Reverse: TCTCATCGAGAAGGATTGGC

vps-29

Forward 1: CTTCAACCTGCCTCATCATCGAG

Forward 2: CTACGAATGCAGTGCTGTCG

Reverse: ACTTGCTCTGTAGGCCATCG

PCR products were separated in 1% agarose gel containing ethidium bromide by electrophoresis in TBE buffer.

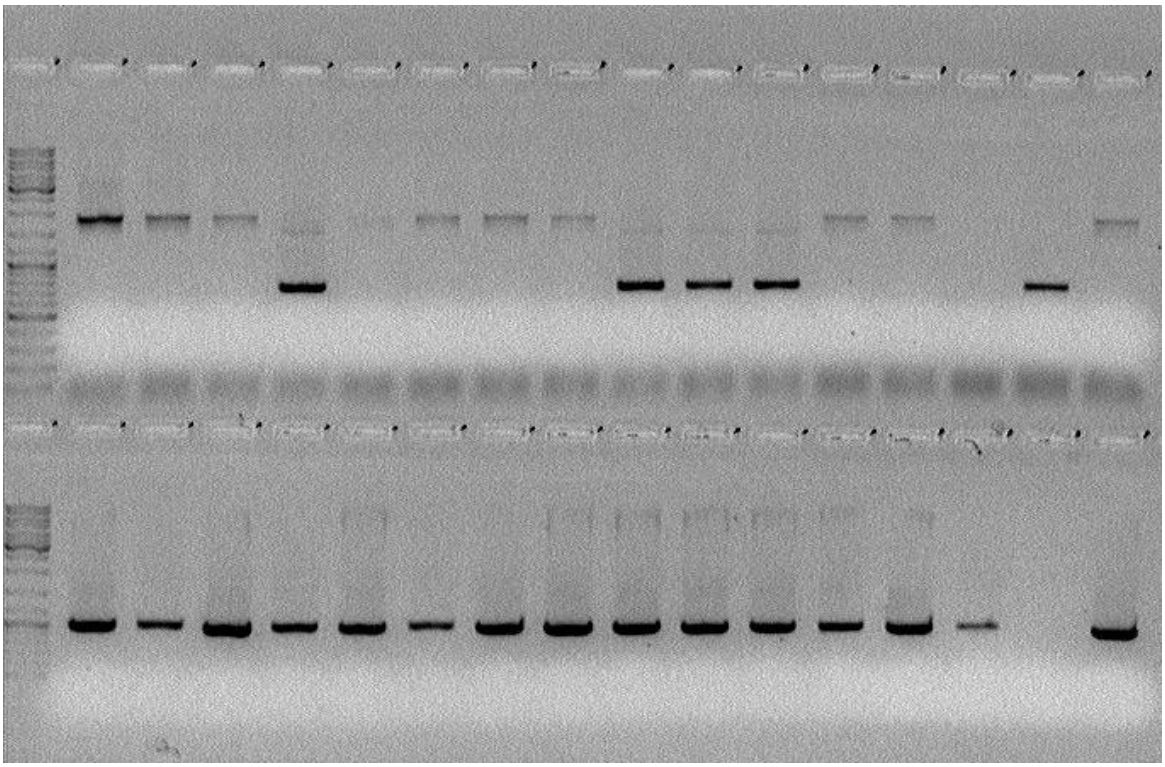

Figure 3: An example of *cup-4* genotyping

An example of the agarose gel with separated PCR products from genotyping animals for *cup-4*. Two different sets of primers and PCR reactions for each individual (sample) were used; PCR product yielded with one set of primers is on the top, the other from the same sample is below it in the same column. Lanes : 1 – molecular standard (PageRuler DNA Ladder Mix, Fermentas), 2-15 – samples, 16 – positive control (*cup-4* homozygote), 17 – negative control (wild type); Rows: top row – set of primers for detection of the deletion allele (when the deletion allele is present, shorter product is made), bottom row – set of primers for detection of the wild type allele (PCR product is made only when wild type allele is present). Heterozygotes are in lanes 5,10,11 and 12, other samples belongs to wild type homozygotes; only sample in the lane 15 cannot be assigned to a genotype because PCR for detection of the deletion allele (top row) yielded no product.

4.3.3 Individual crossing strategies

Some genes required special treatment so we introduced few modifications to general crossing strategy. This applied for *huSi2* transgene, which is located in the same linkage group (II) as the transgene *muIs32*. Also *vps-29* is found on the same chromosome as *cup-4*. *pry-1* mutant strain carried no marker and could not be easily genotyped. Only crossing of *mtm-6 (ok330)* to *cup-4 (ok837)* mutant background had no special requirements so unmodified general crossing strategy could be used.

4.3.3.1 *cup-4* × *huSi2* crossing

huSi2 product is visible only with the use of standard or confocal fluorescence microscopy which demands that the observed worms do not move and ultimately leads to their death, whereas the presence of *muIs32* can be inspected in living animals by stereoscopic fluorescence microscope. I took advantage of this fact to increase my chances to obtain *huSi2* positive progeny:

P: I introduced males into *cup-4* mutant population

F1: I crossed heterozygotic males of the following generation to *huSi2* hermaphrodites

F2: I picked *muIs32* (and *cup-4*) positive worms from their progeny, so only LGII chromosomes with *huSi2* or *muIs32* transgenes, respectively, remained in the population.

F3: In the next generation, I have chosen *cup-4 (ok837)* homozygous hermaphrodites negative for *muIs32* – these were likely to carry two copies of chromosome II with *huSi2* but not *muIs32* unless recombination has happened.

F4: I used their progeny to establish a new *cup-4 (ok837); huSi2* mutant/transgenic line, after I first checked with fluorescence microscope that indeed all worms in the population carry the *huSi2* transgene.

4.3.3.2 *cup-4* × *vps-29* crossing

The crossing strategy in this case was the same as general until the F3 generation. Given that *cup-4 (ok837)* is located on the third chromosome just as *vps-29*, I had to search for recombination event that brought both mutant alleles together into the same copy of the chromosome. According to *http://www.wormbase.org*, genetic distances of *cup-4* and *vps-29* genes are -0.53 +/- 0.003 cM and 1.83 +/- 0.001 cM from the centromere, respectively, yielding |-0.53 cM| + |1.83cM| = 2,33 cM overall

genetic distance between the two genes. This number equals the frequency or probability of recombination between these locuses expressed in percentage.

I thus picked 106 F3 progeny worms from a *cup-4 (ok837)* positive (as determined by genotyping), *vps-29* heterozygous parent. After they laid embryos I genotyped them for both mutant alleles and searched for a parent homozygous in one mutation and heterozygous in the other. Such a worm carries one copy of a recombinant chromosome with both mutations. In F4 generation, I could then easily find the individual with both copies of this chromosome.

4.3.3.3 *cup-4* × *pry-1* crossing

pry-1 (mu38) is a point mutation which cannot be detected by the PCR method I used. Furthermore, mutant strain carried no transgene to detect the positions of the Q neuroblasts. I knew from previous published studies (Korswagen et al., 2002; Maloof et al., 1999) that *pry-1* mutants display defect in QR migration. When I crossed *cup-4* mutation into *pry-1* mutant homozygous strain, I thus first picked *muIs32* positive worms and after they laid enough embryos and larvae have hatched, I genyped them for the presence of *cup-4 (ok837)* allele. I used *muIs32* positive progeny of *cup-4* heterozygotes as a parents for the next generation, genotyped them for *cup-4* and picked *muIs32* positive worms with QR migration defect from the progeny of *cup-4* mutant (to establish *cup-4 (ok837); pry-1 (mu38)* double mutant line) or wild type (to obtain *pry-1 (mu38); muIs32* line) homozygotes. I repeated the last step one or two more times in the next generations and when I found QR phenotype stable, I could consider new mutant/transgenic lines to be established.

4.4 The QL migration assay

I used migration of QL neuroblast to adress the functionality of Wnt signalling. Migration of QL neuroblast is governed by only one Wnt ligand, EGL-20, and is phenotypically very distinctive which makes it useful model of Wnt signalling to study.

To determine the position of Q neuroblasts, I used *C. elegans* strains carrying the *muIs32* transgene, which encodes green fluorescent protein under tissue specific promoter active in one of the daughter cells of both the QL and the QR neuroblasts, ALM and PLM neurons. For standard scoring of the phenotype, non-synchronized population of worms from well grown plate was washed with M9 buffer, pelleted down and 6 – 8 µl

pipetted onto 2% agarose pad on a microscopic slide and covered with cover lid. Agarose contained 10mM sodium azide to paralyze the animals and hence prevent their movements during the experiment. I used the samples for standard wide field fluorescence microscopy (Olympus BX40, magnification 480×, no immersion).

I inspected QL and QR of larvae or adults whose positions of both Q neuroblasts could be determined. Animals were considered to be „wild type“ when their QL neuron was located posterior and QR anterior to vulva. When both QL and QR were positioned anteriorly to vulva, animals were scored as „QL migration defective“. Similarly, animals were considered to be „QR migration defective“ if both QL and QR were located posteriorly from vulva. Only few animals whose QL or QR position was intermediate (in the middle of vulva) and thus could not be assessed to any of the phenotypic class, were excluded from the analysis. Preferred stage for scoring were L3 and L4 larvae, where the positions of neurons and vulva are best visible.

4.5 RNAi

RNAi was performed on 6-well plates by feeding worms with bacteria clones from *Caenorhabditis elegans* genomic library (Kamath et al., 2003). Each of these bacteria clones carries plasmid with a piece of *C. elegans* genome. When induced by IPTG, they synthesize RNA corresponding to the genomic sequence, which triggers RNAi in the worms feed on the bacteria.

Each well has 3 cm in diameter and 3 ml of agar were used per well. Every time, I prepared 2-3 wells with the same RNAi and *C. elegans* strain – at the end, populations of worms from these wells vere mixed and used for phenotype scoring.

Agar was mixed as follows:

for 300 ml (approximately sixteen 6-well plates, 3 ml per one well + four 3 cm plates, 3 ml each):

For 300 ml

0,9 g NaCl

0,75 g peptone (Tryptone)

291,5 ml H_2O

autoclave, cool down to approximately 40 – 50 °C

7,5 ml K-phosphate buffer, pH = 6,0

0,3 ml 1M $CaCl_2$

0,3 ml 1M $MgSO_4$

0,3 ml cholesterol (5 mg/ml in EtOH)

300 μl ampiciline (50 mg/ml)

750 μl tetracycline (5 mg/ml)

300 μl 1M IPTG

A fully grown plate of *cup-4; muIs32* (KN1151), muIs32 (CF700), *huSi2* (KN1312) or *cup-4; huSi2* worms with many adults filled with unhatched embryos were subjected to hypochlorite treatment (described above in section 4.2.1.1) and embryos cultured overnight in 1 ml of M9 buffer to hatch. Without access to nutrition, these larvae are arrested in L1 stage. Following morning, larvae were transferred to 9 cm plates containing bacteria and let to grow for 2 days to L4 stage.

The same day, HT115 strain *E. coli* bacteria from the *C. elegans* genome library carrying L4440 plasmid with various inserts corresponding to fragments of *C. elegans* genome under IPTG-inducible promoter were inoculated from colonies on agar plates or from glycerol stocks into 1,5 ml of LB medium with 1,5 μl of amipiciline and 3,75 μl of tetracycline and incubated overnight in 37 °C with shaking. Next day, 50 μl of bacteria culture were pipetted to each well of 6-well RNAi plate and allow to grow overnight under laboratory temperature.

Finally, L4 larvae were washed with M9 buffer, pelleted down and 3 to 7 individuals were pipetted to each well outside of bacterie lawn (to prevent contamination of the pipette tip and hence other RNAi wells with bacteria).

Worms grew for 4 days after which the first generation was washed with M9 buffer (400 μl per well), worms from wells with the same RNAi setting were mixed together, pelleted down and used for fluorescence microscopy. Animals were scored for penetrance of the QL migration defective phenotype or localization of MIG-14::GFP *huSi2* transgene product on wild type and *cup-4* mutant background. Usually, around 100 animals (90 – 120) were scored for each microscopic slide/RNAi.

Worms grown on bacteria carrying empty L4440 plasmid were used as a negative control; positive control was represented by RNAi against *snx-3*, which gives strong enhancement of the QL migration defect on *cup-4 (ok837)* mutant strain (see chapter 5, section 5.2.3).

4.6 Confocal microscopy

For microscopy, I followed similar procedure as already described for QL migration assay or RNAi – I washed worms from well grown plate with M9 buffer, pelleted them down in a benchtop microcentrifuge. I pipetted approximately 4 to 10 (usually 6-8) µl of pelleted worms onto 2% agarose pad with 10mM NaN_3 created on a microcopic slide. I coverd the sample with a cover lid and used for confocal fluorescence microscopy (Leica TCS SP2 confocal laser scanning fluorescence microscope) to examine the amount and distribution of MIG-14::GFP or CUP-4::GFP. The images from confocal microscopy were taken in Leica software (LAS) and further processed in FIJI (ImageJ) software. All photographes in the same experiment were taken with the same setting of the camera and microscope. Fluorescence images brightness, contrast or other attributes were not additionally adjusted.

4.7 Western blotting

I used western blotting with anti-GFP antibody to detect MIG-14::GFP levels in *huSi2* transgenic strains. To the functionality and specificity of the anti-GFP antibody, I performed the same protocol also with *arIs37; dpy-20* strain, which secretes GFP into its body cavity (Fares and Greenwald, 2001). To ascertain that the protocol was working and loading of samples was equal, I also probed the same samples with anti-tubulin antibody.

As samples for Western blotting, I used worms from four 90 mm fully grown agar plates for each strain. To obtain L1 larvae, I synchronized the population by hypochlorite treatment. I allowed embryos to hatch overnight in M9 buffer and then feed for 5 to 6 hours on new 90 mm plates. Then I washed them 2-3 times with M9 buffer, and one time with TX-114 buffer (25mM Tris-HCl (pH 7,5), 150mM NaCl, 0,5mM $CaCl_2$, 1% Triton X-114). Finally, I transferred them into TX-114 buffer with protease inhibitor (Roche Complete Mini EDTA Free) added and then broke open worm cuticles by placing the tubes into a liquid nitrogen.

I used two different methods how to break the cells to free their content – sonication (4× 15 seconds separated by few minute pauses on ice) or I pre-chilled a pestle and a mortar and grinded the samples in a liquid nitrogen. I collected the powder, left it to thaw on the ice and spun at 4 °C / 1000 g for 3 minutes. I kept the pellet in –70 °C

for later analysis and centrifuged the collected supernatant at 4 °C / max. speed for 30 minutes. Again, I kept the pellet and collected the supernatant. Such samples were stored in –70 °C for later use. Part of each sample was mixed with 4× concentrated Laemmli buffer (62,5mM Tris (pH 6,8), 2% SDS, 10% glycerol, 5% mercaptoethanol, traces bromophenol blue), heated to 96 °C for 4 minutes and stored in –20 °C for later use for Western blots.

In selected samples, before addition of the Laemmli buffer I measured the protein concentration by the method of Bradford: 5 µl of the sample was mixed with 995 µl of the Bradford solution, incubated for 2-3 minutes and absorbance measured on the spectrophotometer. Absorbance was compared to BSA standards with known concentrations and the actual concentrations of proteins in samples were calculated.

I used SDS electrophoresis (Bio-Rad) on 10 % polyacrylamide gel (5 ml separation + 2 ml stacking gel) to separate the proteins:

10% separation gel (for 10 ml / 2 gels):
2,5 ml 1,5M Tris (pH 8,8)
3,3 ml 30% acryl-bisacrylamide mix
100 µl 10% SDS
100 µl 10% ammonium persulphate
TEMED (2 µl per gel)
4 ml of distilled H_2O

5% stacking gel (for 4 ml / 2 gels):
0,5 ml 1M Tris (pH 6,8)
0,67 ml 30% acryl-bisacrylamide mix
40 µl 10% SDS
40 µl 10% ammonium persulphate
TEMED (2 µl per gel)
2,7 ml of distilled H_2O

Running buffer:
25mM Tris
192mM glycin
0,1% SDS

Proteins from were transferred from the gel onto nitrocellulose membrane using semi-dry or wet blot (BioRad; blotting buffer: 25mM Tris, 192mM glycin, 20% methanol). Membrane was incubated for 5 minutes in amidoblack to confirm that proteins were succesfully transferred. The membrane was subsequently blocked for 1 hour in 3% milk in TBST buffer (ph 7,4; composition:10mM Tris-Cl, 150mM NaCl, 0,05% Tween 20). Primary antibody (anti-GFP (mouse): 0,4 mg/ml, Roche; anti-tubulin (mouse): 5,5 mg/ml, Sigma Aldrich) was diluted at 1:5000 ratio in 3% milk in TBST buffer and incubated with the membrane overnight at 4 °C.

Next day, I washed the membranes three times with TBST (briefly, 10 minutes, 15 minutes) and incubated them for 1 hour with Horse reddish peroxidase-conjugated secondary antibody (HRP-conjugated horse-anti-mouse) diluted to final 1:10 000 ratio. Finally, membranes were washed again with TBST 3 times. I pipetted 1,5 ml of both peroxide and enhancer (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific) onto each membrane and incubated 5 minutes. The substrate was transformed by HRP on antibody labeled proteins to a luminiscent product, so when the membrane was coverd with SARAN and exposed to photographic film (AGFA), bands of proteins could be detected.

4.8 Rescue assay

To determine the tissue specificity of CUP-4 function in Wnt signalling, we decided to express wild type CUP-4 on *cup-4* mutant background under various tissue-specific promoters. This approach was succesfuly used in previous studies to identify the site of action of several proteins involved in Wnt signalling in *C. elegans.*

We selected following promoters:

Pegl-20	for expression in EGL-20/Wnt producing cells
Punc-122	for expression in coelomocytes
Pegl-17	for expression in signal receiving cells (Q neuroblasts)

4.8.1 *cup-4* cloning and constructs

For rescue assay, we first had to isolate wild type *cup-4* cDNA and insert it behind the selected promoter into the appropriate vector. I isolated total RNA from the Bristol N2 wild type *C. elegans* strain and prepared cDNA of all (m)RNAs in the sample. Then, *cup-*

4 cDNA was amplified by PCR with specific primers and *cup-4* sequence inserted into pJET1.2 cloning vector.

4.8.1.1 Isolation of RNA

Worms of *cup-4 (ok837); muIs32* (KN1151) strain were collected with M9 buffer, pelleted and washed with the same buffer three times. After the last wash, around 40 µl of pellet was left. I added ten times volume of the Trizol reagent (Invitrogen) and froze microtubes in liquid nitrogen. Next, samples were incubated at 65 °C for 15 minutes. Then I added 80 µl of chloroform , vortexed and left 3 minutes at room temperature before centrifugation for 15 minutes at max. speed/4 °C. Upper fraction was collected, to which 0,8 volume of isopropanol was added, vortexed and left on the table at laboratory temperature for 10 minutes. The mixture was centrifuged for 10 minutes at max. speed/4 °C. I discarded the supernatant, washed pellet with 500 µl of EtOH, briefly spinned and discarded supernatant. The pellet was left to dry-out in an open microtube and then dissolved in DEPC water. The concentration of RNA was measured on NanoDrop and samples stored at –70 °C for later use.

4.8.1.2 Synthesis of cDNA

SuperscriptIII cDNA synthesis system (Invitrogen) was used. Reaction was mixed as followed:

3 µl RNA (around 6-8 µg)

1 µl oligo-dT or random hexamer primers

1 µl dNTPs

8 µl DEPC water

This mixture was incubated at 65 °C for 5 minutes followed by 1 min. incubation on ice.

5 µl reverse transcriptase buffer

1 µl 0,1 M dithiotreitol

1 µl RNAse inhibitor

1 µl SuperscriptIII reverse transcriptase

The reaction mixture was mixed by pipetting up and down and reactions with random primers additionally incubated for 5 minutes at laboratory temperature. Both reactions (with oligo-dT and random primers) were then incubated for 1 hour at 50 °C. Finally, reverse transcriptase was inactivated by heating to 70 °C for 15 minutes, RNA removed by adding 1 µl of *E. coli* RNAse H (incubated 20 minutes at 37 °C) and samples stored in –70 °C for later use.

4.8.1.3 Amplification of *cup-4* cDNA

PCR reactions were mixed and ran as described for genotyping. First, presence of the *cup-4* cDNA was tested with a set of primers from the inside of the coding sequence of the gene. PCR product was detected on 1 % agarose TBE gel with EtBr. After the presence of *cup-4* transcript was confirmed, I amplified the whole 1.3 kbp transcript using the high fidelity Phusion DNA polymerase (Thermo Scientific) in the HF buffer supplied by the manufacturer.

Primers used for cDNA amplification contained restriction sites for subsequent cloning into plasmids and their sequences were as follows:

Forward: TGGCCAATGAAAATTATAATATTTGTTTGTTTTATATTG
(complementary to the first exon of the *cup-4* gene and containing adapter with MscI restriction site (underlined))

Reverse: GAGCTCTCACAATAAAAGGCAAACGAAAAAC
(complementary to the seventh (last) exon of the *cup-4* gene and containing adapter with SacI restriction site (underlined))

4.8.1.4 Plasmid constructs

cup-4 cDNA was inserted into pJET1.2 *E. coli* plasmid (Fermentas) by CloneJET™ PCR Cloning Kit and transfected into TOP10 *E. coli* strain (later DH-5α strain was used for transfections):

100µl aliquot of bacteria was thawed on the ice, 1 µl of DNA added and incubated for 30 minutes on the ice. After that, bacteria were heat-shocked by placing the microtube into 42 °C for 1 minute followed by adding 900 µl of cold LB medium. Microtube

with bacteria stood 2-3 minutes on the ice before it was transferred to the pre-heated block for incubation for 1 hour in 37 °C with shaking (600 rpm). Finally, 100 µl of grown bacteria culture were spread over 9,4 cm agar plate with ampiciline (50 µg/ml) in a Petri dish. The rest (900 µl) of the bacteria culture was pelleted down in a benchtop microcentrifuge (max. speed/30 sec.) and 800 µl of supernatant discarded. Pelleted bacteria were resuspended and put on agar plate as well. Petri dishes with bacteria were sealed with Parafilm and cultivated in a plastic bag in thermostat (37 °C) overnight.

We decided to additionaly insert GFP coding sequence into the *cup-4* cDNA after amino acid 238, so we could later examine CUP-4 intracellular localization and colocalizations with other members of the Wnt secretion or signalling machinery. This way of CUP-4 tagging was formerly proved to be functional since it can rescue *cup-4* defect in coelomocyte endocytosis (Patton et al., 2005). We therefore introduced additional restriction sites into *cup-4* cDNA by mutagenic PCR. Following primers containing inserts with EcoRV and XhoI restriction sites were used:

Forward:
GGATTTCAAAATCCAAAACGTCGATATCGCTAGCCTCGAGTCAAGCTCAGCACTTTACTA
(EcoRV restriction site underlined, XhoI restriction site double-underlined)
Reverse:
TAGTAAAGTGCTGAGCTTGACTCGAGGCTAGCGATATCGACGTTTTGGATTTTGAAATCC
(EcoRV restriction site underlined, XhoI restriction site double-underlined)

Compatible EcoRV and SalI restrictions sites were joined to the *gfp* gene without introns in the pJET1.2 vector by PCR with primers with 5‘ overhangs that contained the restrictions sites. Primers were as followed:

Forward (EcoRV): GATATCATGAGTAAAGGAGAAGAACTTTTCACTGGAG
(EcoRV restriction site underlined)
Reverse (SalI): GTCGACTTTGTATAGTTCATCCATGCCATGTG
(SalI restriction site underlined)

Modification of both *cup-4* and *gfp* were confirmed by sequencig with pJET1.2 sequencing primers:

Forward: CTGCTTTAACACTTGTGCCTG
Reverse: CTACAACGGTTCCTGATGAGG

For insertion of *cup-4* under *egl-20* promoter, I excised modified *cup-4* from pJET1.2 by MscI and SacI restrictases. Next, I opened *Pegl-20* containing pPD49.26 plasmid behind *egl-20* promoter with BamHI, treated the cohesive ends with T4 DNA polymerase (Promega) to generate blunt ends and finally digested the plasmid with SacI. I isolated the fragments of interest on 1% agarose gel in TBE or TAE buffer, excised and extracted them from the gel with a Gel extraction kit (Geneaid). I prepared 20 μl ligation mixture (T4 DNA ligase, Fermentas) and transfected ligation product into TOP10 or DH-5α *E. coli* bacteria. Transfected bacteria were spread over 9 cm LB agar plate with ampiciline and cultured overnight at 37 °C. Next day, colonies were analyzed by colony PCR for the presence of the construct. Finished pPD49.26 *Pegl-20::cup-4* construct was then opened by EcoRV and XhoI restrictases to insert *gfp*.

gfp sequence was excised from pJET1.2 by EcoRV and SalI and inserted into pPD49.26*::egl-20p::cup-4* opened by EcoRV and XhoI restriction endonucleases. I analyzed obtained clones by colony PCR (sequencing primers shown below) for the presence of the correct pPD49.26*::egl-20p::cup-4::gfp* construct and after positive result, I have chosen four colonies, inoculated them into LB medium with ampiciline and isolated plasmid construct DNA by MiniPrep (Macherey-Nagel). I determined DNA concentration by NanoDrop and mixed DNA sequencing reactions according to instructions of Laboratory of DNA sequencing at Faculty of Sciences, Charles University in Prague, where constructs were sequenced. From sequences, *egl-20p – cup-4*, *cup-4 – gfp* and *gfp – cup-4* boundaries were aligned and checked against the expected sequence in BioEdit. Primers used for sequencing were as follows:

Pegl-20/cup-4 border: CAATCGTCTTCTTAACCAGG
(anneals to *egl-20* promotor)

cup-4/gfp border (5‘): TTTCAACATCAACAATGGCTG
(anneals to *cup-4* exon 4)

gfp/cup-4 border (3‘): CAGCATAGACCAAGTAGAGC
(anneals to *cup-4* exon 5)

4.8.2 Transgenesis

After confirmed to be correct, *Pegl-20::cup-4-gfp* construct was sent to MSc. Masako Asahina-Jindrová, Ph.D. (Laboratory of Molecular Genetics of Nematodes, Biology Center, ASCR) who injected it into gonads of wild type N2 strain hermaphrodites along with *Pmyo-2::tdTomato* co-injection marker. *tdTomato* is then expressed in pharynxes of transgenic animals, where it can be detected by its red fluorescence signal. The resulting transgene is not integrated into a chromosome segregates randomly during each mitotic division. Therefore it is lost in part of the progeny in each generation. Hence, I had to pick several *dtTomato* positive animals every few weeks to preserve the transgenic strain.

5 Results

5.1 QL migration defect of *cup-4* mutants is sensitive to cultivation temperature

It has been recently shown that a mutation in the *cup-4* gene affects the EGL-20/Wnt-dependent migration of the Q neuroblasts, albeit only in a small fraction of population (Silhankova et al., 2010). In this study the *C. elegans* strains were cultivated at the standard temperature of 20°C. There are some indications that the Q cell migration phenotype could be sensitive to temperature (M. Macůrkova, unpublished). I therefore wondered whether the penetrance of the QL migration defect of *cup-4* mutant worms would be affected by changes in cultivation temperature.

For this purpose, I maintained two populations of the *cup-4 (ok837); muIs32* (KN1151) *C. elegans* strain in thermostat set to 20 or 15°C, respectively. After several generations grown in given temperature, I counted the penetrance of the QL phenotype. I found that the QL migration defect is more penetrant in animals cultivated at lower temperature (Figure 4) – the observation I also made on *vps-29; muIs32* strain, which were placed in 15 °C and compared to the same strain from 20 °C. Examples of both wild type and QL migration defective animals are shown in the Figure 5. Treatment with hypochlorite, insufficient amount of bacteria and lack of enough space (over-grown plate), i. e. any kind of stress, seemed to have the same effect, although these are just personal observations and data were not collected and are thus not shown. Since lower phenotypic penetrance is more advantageous while searching for its further enhancement, I used non-synchronized animals cultivated in 20 °C for all the experiments, if not stated otherwise. The penetrance of the QL migration defect also seemed to be more fluctuating and less stable under various forms of stresses, so I tried to avoid them.

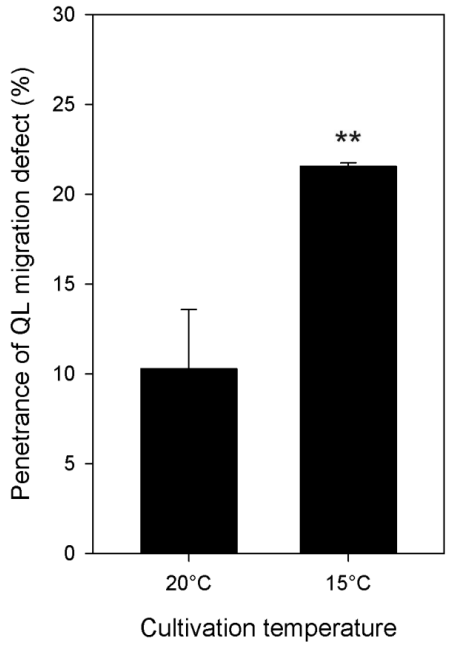

Figure 4: Dependence of *cup-4* QL migration defect on temperature

Penetrance of QL migration defect in *cup-4 (ok837); muIs32* cultivated under different temperatures. Population from 15 °C exhibited more penetrant defect than population cultivated under 20 °C. Bars are mean values from 3 independent experiments; error bars represent standard deviations. Number of animals in each experiment varied from 77 to 167, but usually was around 120.

p-value = 0,0041, unpaired *t*-test, equal variances.

(* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

Figure 5: QL vs. WT phenotype (see next page)

Confocal microscopy side view images of QL migration defective and wild type worms carrying *muIs32* transgene. This transgene encodes *gfp* sequence behind *mec-7* promoter (*Pmec7::gfp*) which cauese *gfp* to be expressed in several neurons. These inculde two ALM neurons with long anterior projections in the middle of the body (only one ALM neuron is visible on images; the next is hidden behind it), two PLM neurons with long anterior and short posterior axons in the tail and, finally, in two Q neuroblasts daughter cells. AVM neuron (marked by asterisk) from the QR lineage is located anterior – in the middle of the body near PLM – in both the wild type and QL phenotype. PVM (marked by arrowhead) is located in the posterios half of the body between ALM and PLM in wild type. This position is specified and requires functional EGL-20/Wnt signalling. Thus, in mutants in some positive regulator of Wnt signalling where Wnt signalling is compromised, PVM migrates in the default anterior direction, just like QR near PLM. Then, the situation, when four GFP-positive cells can be seen in the mid-body (anterior to vulva), two PLM neurons in the tail and no other GFP posive cell is located between them (4-0-2; wild type 3-1-2) is referred to as „the QL phenotype“. In a rarer situation, when some negative regulator of Wnt signalling (like Axin homologue *pry-1*) is inactivated, both QL and QR daugter cells are found in posterior position (not shown). This phenotype (2-2-2) is referred to as „the QR phenotype“.

Images are maximal projections of 26 (QL) or 32 (WT) sections taken with *z* steps of 0,49 µm. Representative specimens of each phenotype are shown. Scale bar represents 50 µm and applies for all images

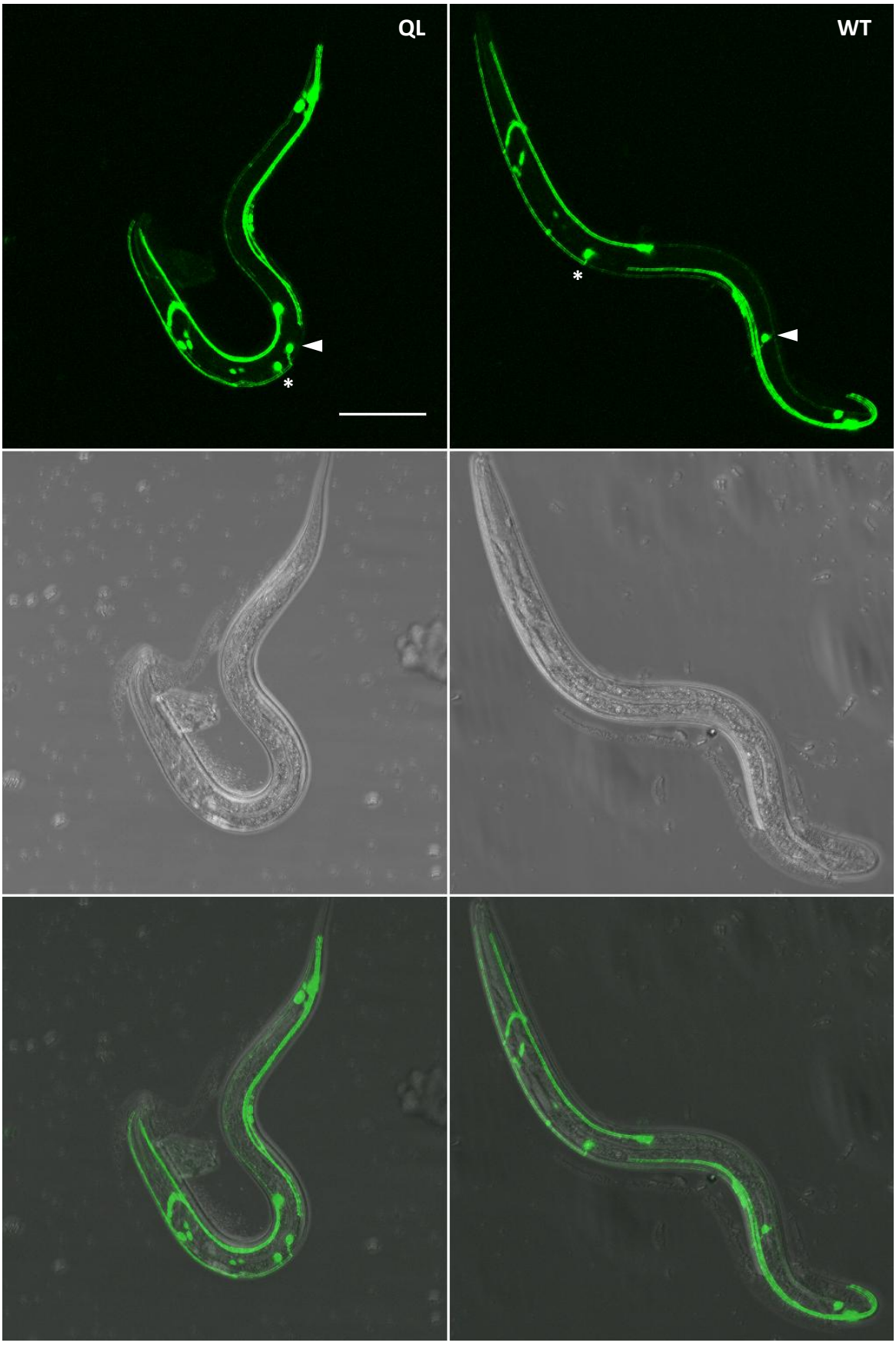

Figure 5: QL vs. WT phenotype

5.2 Genetic interactions of *cup-4*

5.2.1 CUP-4 acts upsream of PRY-1/Axin

Gene *pry-1* encodes a *C. elegans* homologue of Axin (Korswagen et al., 2002), part of the destruction complex which, in canonical Wnt signalling cascade, targets β-catenin for degradation. This is also the case in neuroblasts of the Q-lineage, where PRY-1 activity is required to prevent expression of the Hox gene *mab-5* in the right neuroblast QR (Maloof et al., 1999). In *pry-1 (mu38)* mutants, β-catenin BAR-1 is not degraded and can enter nucleus in both QL and QR neuroblasts, where it activates transcription of *mab-5*, which in turn leads to posterior migration of QL and, in contrast to wild type situation, also of QR (Korswagen et al., 2002; Maloof et al., 1999). However, the penetrance of this phenotype is not absolute, since there is another Axin homologue, AXL-1, which can act partially redundantly with PRY-1 in the Q neuroblasts in targeting β-catenin to degradation and hence repressing *mab-5* expression (Oosterveen et al., 2007). Overexpression of *pry-1* leads to inhibition of *mab-5* expression and to QL migration defect (Korswagen et al., 2002).

By crossing *muIs32* transgene into *pry-1 (mu38)* mutant background, I confirmed these results (Figure 6, left). Although the strain I used showed somewhat higher penetrance of QR migration defective than stated in (Korswagen et al., 2002) but in agreement with data from (Maloof et al., 1999), the important fact – nature of the defect – remains the same, i. e. abnormal posterior migration of QR.d and wild type posterior migration of QL.d suggesting ectopic activation of *mab-5* in QR. However, I did not investigate *mab-5* expression in QR nor QL neuroblast lineage.

Here, I show that *pry-1 (mu38); cup-4 (ok837)* double mutants I have prepared also exhibit QR phenotype with similar penetrance as observed in *pry-1 (mu38); muIs32* single mutant (Figure 6, left) but not the QL phenotype of *cup-4 (ok837)* single mutant (Figure 6, right). This means that CUP-4 acts upstream from PRY-1 in Wnt signalling.

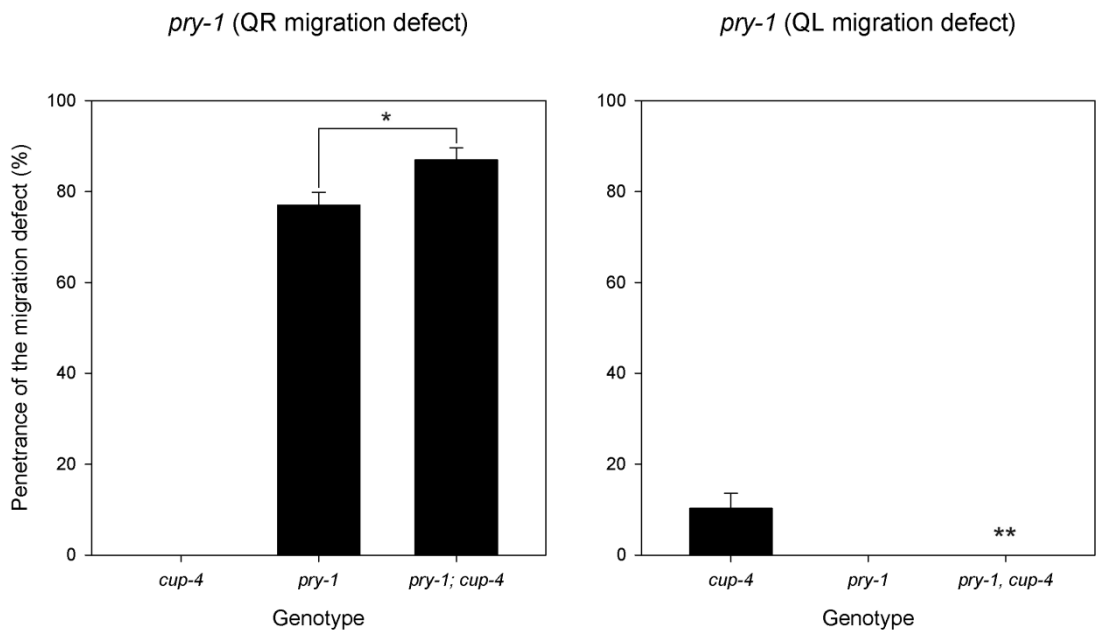

Figure 6: Genetic interaction between *cup-4* and *pry-1*

Genetic interactions between *cup-4* and *pry-1*. *cup-4* mutation causes migration defect of the QL when when it is present alone but not when it is together with the *pry-1 (mu38)*. Instead, the highly penetrant QR migration defect was observed in *pry-1 (mu38); cup-4 (ok837)* double mutants suggesting that PRY-1 functions downstream of CUP-4 in the Wnt signalling. The deficiency in the Wnt signalling caused by *cup-4* mutation is thus overruled by the downstream hyperactivation of the Wnt signalling cascade by the elimination of PRY-1. The difference between the penetrance of the QR phenotype between *pry-1* single mutants and *pry-1; cup-4* double mutants is of low significance and will be discussed later.

Bars represent mean values of three (*pry-1* and *cup-4; pry-1*) or four (*cup-4*) independent experiments (n = 3-4), each containing usually 90 (for *pry-1*), 120 (for *cup-4; pry-1*) or 77 to 167 (*cup-4*) animals; error bars represent standard deviations. All strains also contain the *muIs32* transgene.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, unpaired *t*-test (compared to *pry-1 (mu38); muIs32* strain for QR or to *cup-4; muIs32* for QL)

5.2.2 DPY-23 and APA-2 subunits of AP2 clathrin-dependent endocytosis adaptor complex

cup-4 mutants show strongly reduced levels of PI-4,5-P_2 (Patton et al., 2005) which recruits adaptin AP2 to the cytoplasmic membrane (Höning et al., 2005). AP2 binding is necessary for the clathrin-mediated endocytosis of Wls from the plasma membrane and it is thus important for efficient EGL-20/Wnt signalling (Pan et al., 2008a; Yang et al., 2008). A possibility thus occurs, that this could be the mechanism how CUP-4 affects EGL-20 signalling. To test this or to determine whether CUP-4 functions downstream or upstream of clathrin-dependent endocytosis of MIG-14/Wls from plasma membrane

of EGL-20/Wnt producing cells, I performed RNAi against two components of AP2 adaptor complex. I have chosen *dpy-23*, which encodes the AP2 μ subunit, and *apa-2*, which encodes the α subunit.

I performed RNAi against these genes on *huSi2* and *cup-4 (ok837); huSi2* strains to investigate the distribution and localization of MIG-14::GFP fusion protein encoded by *huSi2* transgene in wild type and *cup-4* mutant background. MIG-14::GFP was reported to be homogenously distributed in plasma membrane, when AP2 function is impaired or missing (Pan et al., 2008a; Yang et al., 2008). I largely confirmed these results (Figure 7, left) and I found, that *cup-4; huSi2* exhibit the same phenotype (Figure 7, right).

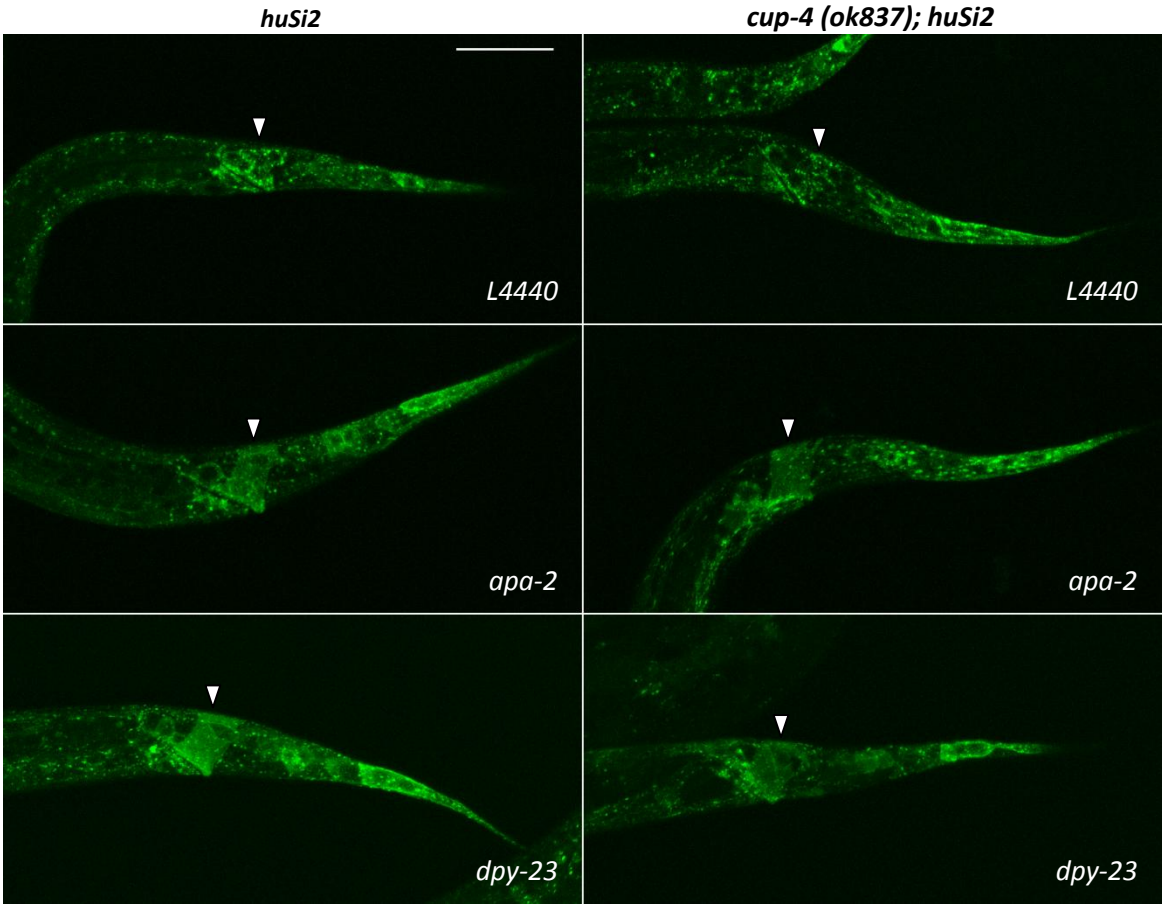

Figure 7: Localization of MIG-14::GFP on RNAi against AP2

Confocal microscopy images of MIG-14::GFP encoded by *huSi2* transgene in wild type and *cup-4* mutant background. Knock down of *apa-2* or *dpy-23* AP2 subunits causes membrane localization of MIG-14::GFP in both strains whereas MIG-14:GFP localization is more puncate on control *L4440* RNAi (arrowheads point to EGL-20/Wnt producing cells where the difference is best visible). Strain genotype is quoted at the top of the columns, RNAi is indicated in the lower right corner of each image. In all images anterior is left, dorsal is up. Representative individuals for each condition are shown. Images are maximal projections of 10 (for *L4440* and *apa-2* RNAi), 25 (*huSi2/dpy-23*) or 30 (*cup-4; huSi2/dpy-23*) subsequent photographs in *z* taken with *z*-steps of 0,2035 μm. Scale bar represents 20μm and applies for all images. Setting of the microscope was the same for all images. Maximal projections were made in FIJI software. False colours were assigned to channels using the same software, otherwise no modifications were made.

Both *cup-4; huSi2* grown on negative control L4440 RNAi show punctate distribution of MIG-14::GFP. These results indicate, that CUP-4 is not necessary for MIG-14:GFP to reach the cytoplasmic membrane. Moreover, localization of MIG-14::GFP in animals grown on bacteria carrying L4440 empty RNAi vector suggests that the internalization of Wntless from cytoplasmic membrane in the *cup-4* mutants is also not affected.

5.2.3 *cup-4* QL migration defect is enhanced by elimination of retromer components

Retromer is required for an efficient retrieval of Wntless back from endosomes to the trans-Golgi reticulum, hence preventing its degradation in lysosomes and making it available for participating on another round of Wnt secretion. In case the retromer function is lacking, the amount of Wntless becomes limiting for Wnt secretion and signalling (Belenkaya et al., 2008; Franch-Marro et al., 2008; Pan et al., 2008a; Port et al., 2008; Yang et al., 2008).

I wondered, if *cup-4* can enhance the penetrance of QL-migration defect of mutants in various components of retromer. I thus tried to cross *vps-29*, a component of cargo-selective subcomplex, to *cup-4* mutant background (see Materials and Methods, section 4.3.3.2). Out of 106 F3 animals, 96 survived and could be genotyped. Between them, I found 3 possible recombination events. Their double mutant progeny, however, turned out not to be viable, so I was forced to perform RNAi instead.

Although RNAi against *vps-29* was reported to be inefficient (Coudreuse et al., 2006), i. e. it did not exhibit any Wnt-related defects, and in my hands it showed normal phenotype on wild type CF700 (*muIs32*) strain, it significantly increased penetrance of QL migration defective phenotype of *cup-4* mutant worms (Figure 8). This suggests, that both CUP-4 and VPS-29 participate on the same process in the Wnt signalling, although it does not distinguish between CUP-4 and VPS-29 acting subsequently or together in the same pathway or in two parallel pathways.

With this in mind, I performed RNAi against another component of retromer cargo-selective subcomplex, *vps-35*. This protein is more required for Wntless recycling and Wnt signalling, since its single mutant display highly penetrant defect in EGL-20/Wnt-dependent migration of QL neuroblasts (Coudreuse et al., 2006). However, in my

hands, RNAi against *vps-35* did not lead to any enhancement of the QL migration defect caused by *cup-4* mutation alone (Figure 8). I discuss this result later.

Next, I performed RNAi against the sorting nexin 3 gene *snx-3*. The function of *snx-3* is likely to be in recruiting of retromer cargo-selective subcomplex to Wls containing vesicles through binding of phosphatidylinositol-3-phosphate (Harterink et al., 2011b; Silhankova et al., 2010). By comparing with *snx-3* RNAi on wild type CF700 (*muIs32*) strain, I found that *snx-3* knockdown alone causes no QL migration defect but it strongly enhances the QL phenotype of *cup-4* mutants (Figure 8). This leads to conclusion that again, *snx-3* is likely to function in the same process as *cup-4* in EGL-20/Wnt signalling.

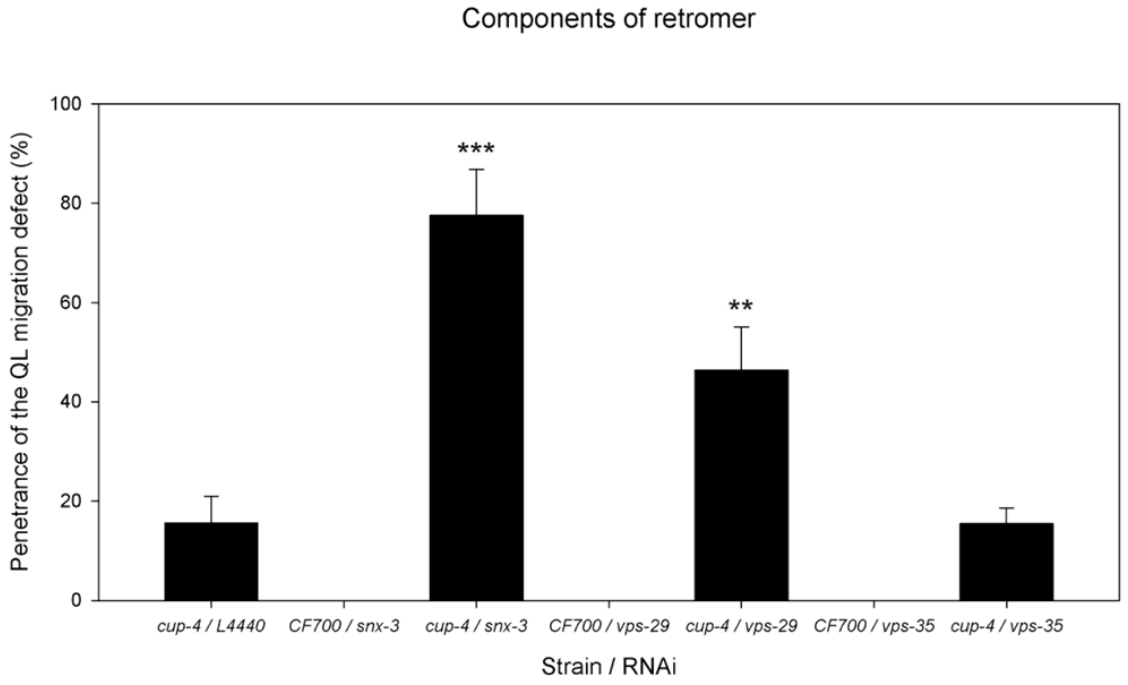

Figure 8: Genetic interactions of *cup-4* and components of retromer

Genetic interactions between *cup-4* and various components of the retromer complex as determined by RNAi. There is a very strong interaction between *cup-4* and the sorting nexin *snx-3*. This gene was used later as a positive control for all other RNAi experiments. Conflicting results were obtained for various subunits of the retromer cargo-selective subcomplex: whereas weaker, but still strong enhancement of *cup-4* QL migration defect was observed in *vps-29* RNAi suggesting involvement of both CUP-4 and VPS-29 in the same process, no interaction between *vps-35* and *cup-4* was detected. Knockdown of none of the genes tested caused the QL migration defect on its own, i. e. when performed on the wild type background. Since *vps-35* was previously reported to display a strong phenotype and since its protein product functions in the same complex together with VPS-29, it is very likely that the knockdown of *vps-35* was inefficient.

Bars represent mean values of several independent experiments (n = 4 for *vps-29*, n = 3 for *vps* and n = 7 for *cup-4* and *snx-3*), each containing usually 90 to 100 animals (N = 90 – 100); error bars represent standard deviations.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, unpaired *t*-test (compared to L4440 control RNAi on *cup-4 (ok837); muIs32* strain)

5.2.4 CUP-4 functions in the same process as MTM-6 myotubularin 3-phosphoinositide phospatase

Myotubularin lipid-phosphatases are also another key – although not known for a long time – players in recycling Wntless for repeated use. From all *C. elegans* myotubularins, the active phosphatase MTM-6 and its binding parner, inactive phosphatase MTM-9, are the most important for EGL-20/Wnt signalling (Silhankova et al., 2010).

Interaction of *cup-4* with *mtm-6* has already been investigated, but the strain was lost. I prepared the *cup-4 (ok837); mtm-6 (ok330); muIs32* double mutant/transgenic strain again and counted percentages of worms with defect in migration of QL.d in double and both single mutants, respectively. I confirmed previous results and found a strong enhancement of phenotype penetrance of *cup-4 (ok837)* mutant by introducing *mtm-6 (ok330)* mutant allele (Figure 9). Again, as in the case of *vps-29*, this suggest CUP-4 and MTM-6 acting in the same process in Wnt signalling, but without distinguishing between both proteins participating on the same or two parallel pathways.

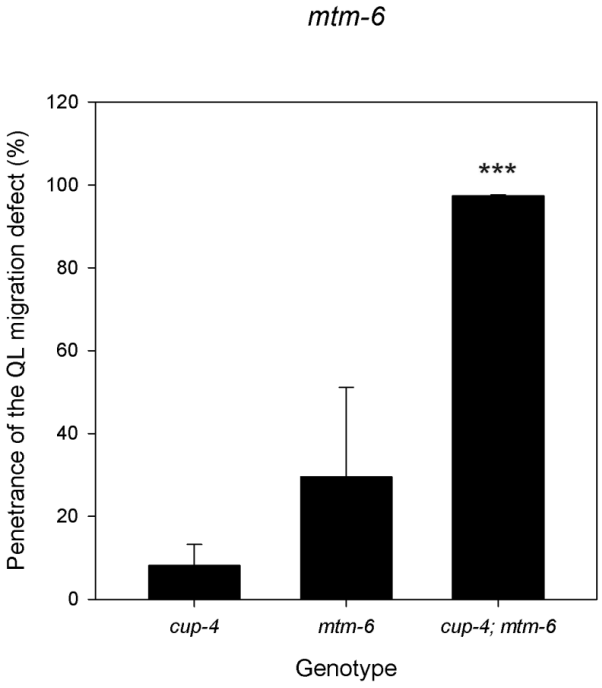

Figure 9: *cup-4* strongly interacts with *mtm-6*
Genetic interaction of *cup-4* and *mtm-6. mtm-6* strongly enhances *cup-4* mutant phenotype and *vice versa*. Single mutant strains carrying *cup-4 (ok837)* or *mtm-6 (ok330)* mutant allele as well as double mutant strain obtained by their crossing were kept in the same conditions and scored for penetrance of the QL migration defect using wide field fluorescent microscopy. Bars represent mean values of three to four independent experiments (n = 3-4), each containing 90 to 123 animals (N = 90 – 123); error bars represent standard deviations. Wide standard deviation interval for *mtm-6 (ok330); muIs32* strain is caused by great variation among single replicates, e.g. one time, animals from very densely grown plate were used for microscopy which resulted in more than two times higher penetrance than in preceding two replicas of the experiment (approx. 60 vs. 25 %). Such stressing situation could enhance penetrance of QL migration phenotype on their own, although this hasn't been proved. However, it did not affect the conclusion from this experiment so I did not exclude this result. All strains also contained *muIs32* transgene.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, unpaired *t*-test (compared to *cup-4 (ok837); muIs32*)

5.2.5 Phospholipases

It seems that tight regulation of 3-phosphoinositides is very important for efficient recruiting of retromer and retrieving of Wls from endosomes to the trans-Golgi reticulum. We wondered whether other enzymes, which can modify amount and distribution of phosphoinositides in membranes, have some effect on Wnt signalling. Actually, hyperactivation of phospholipase C was proposed as the reason of diminished PI-4,5-P_2 levels and impaired endocytosis of *cup-4* mutants (Patton et al., 2005). If true and if the same defect of endocytosis is also responsible for obsereved defect in Wnt signalling, I would expect that elimination of phospholipase activity would rescue the *cup-4* Wnt phenotype.

Among *C. elegans* phospholipases, there are six phospholipases C, which can cleave off the group bound on the third carbon atom of phospholipid glycerol chain. Among these, several exhibit some phenotypes that could be potentionally relevant for Wnt signalling (summarized in Table 4)

Out of the listed genes, all but *egl-8* and *ipla-1* are available in our *C. elegans* genome library for RNAi. Thus, I performed RNAi for all but these two genes on *cup-4* mutant and wild type strains, respectively, and observed the penetrance of QL migration defective phenotype. RNAi against pld-1, a phospholipase D homologue that cleaves phosphatidylcholine and thus should not directly affect phosphatidylinositol phosphate levels, was included as a control.

Among four *C. elegans* phospholipases C (*plc-1, plc-2, plc-3, plc-4*), I did not find any rescue nor enhancement of *cup-4* QL migration defect (Figure 10). Small enhancement seen in graph for *plc-4* is not significant. I found small enhancement with low significance in phospholipase C-like *pll-1* (Figure 11, left). Knock down of phospholipase D (*pld-1*) had no effect (Figure 11, right). Knock-down of any phospholipas alone did not cause QL migration defect on CF700 (*muIs32*) wild type control strain.

There still remains the possibility that RNAi could have been uneffective or insufficient, especially due to persisting redundant functions of related enzymes. This apply especially for numerous phospolipases C. To rule this out, oune would have to knock down all the phospholipases at the same time. However, I was told that performing simultaneous RNAi against multiple genes is not reliable method, although I found cases in literature where such an assay was done – e. g. (Xue et al., 2003).

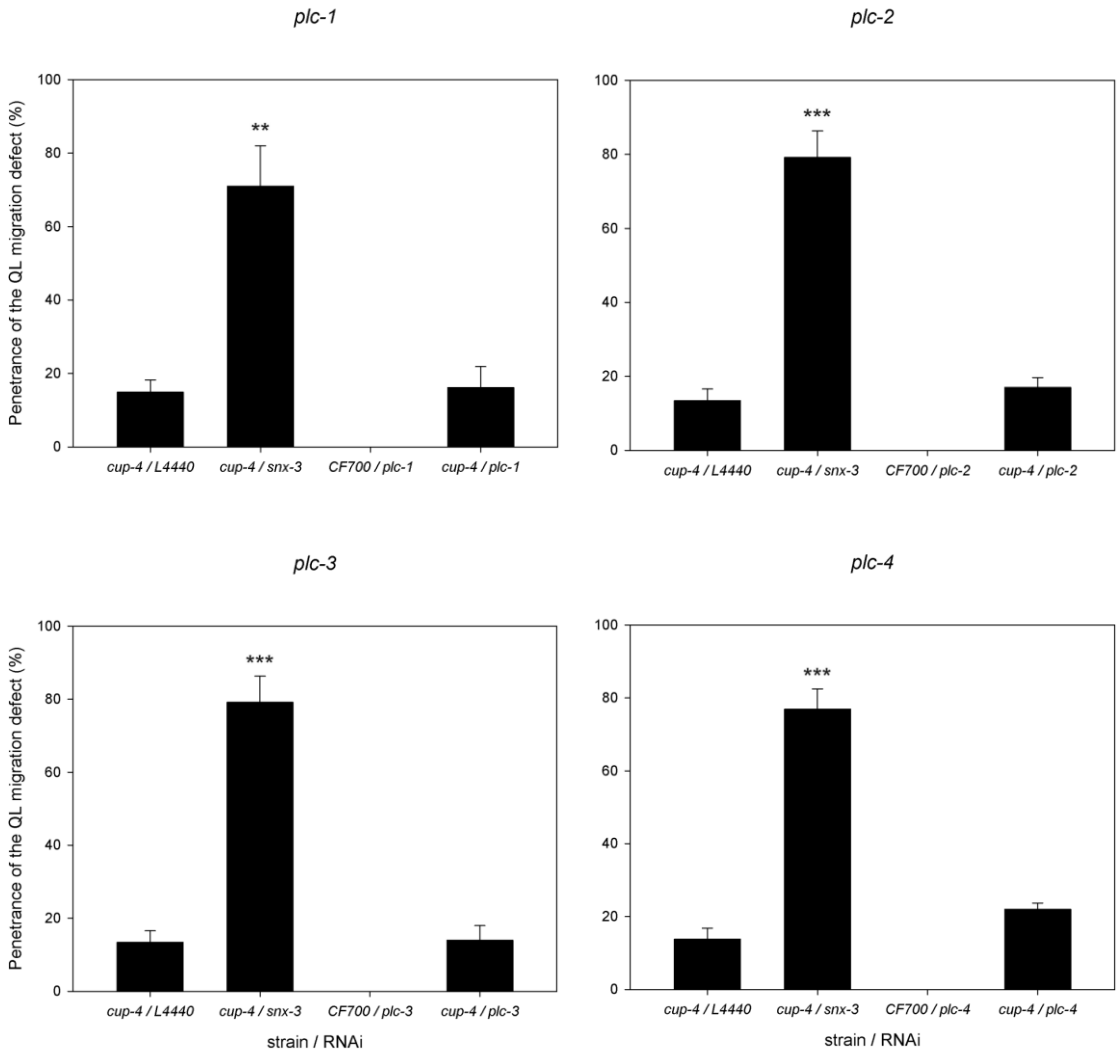

Figure 10: Interaction between *cup-4* and *C. elegans* phospholipases C

Genetic interactions between *cup-4* and various *C. elegans* phospholipases C *plc-1*, *plc-2*, *plc-3* and *plc-4* by performing RNAi on *cup-4 (ok837)* mutant background. There was some small enhancement when *plc-4* was knocked-down, but the difference between *plc-4* and control RNAi was not significant, largely due to large variations between results of individual experiments. Knock-down of any phospholipase C did not cause QL migration defect on CF700 (*muIs32*) control strain. For all genes tested, RNAi against *snx-3* is also shown as a positive control. Bars represent mean values of at least three independent experiments (n = 3), each containing usually from 90 to 100 animals (N = 90 – 110; except for 3 cases in *plc-1* and *plc-2*, where N ranged sometimes from 63 or 70 and one in *plc-3*, where N was 110); error bars represent standard deviations. Since all genes could not always be tested in the same batch, controls are slightly different for each gene; that is why separate bar plot with separate controls are shown for each gene.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, unpaired *t*-test (compared to L4440 RNAi on *cup-4 (ok837); muIs32* strain)

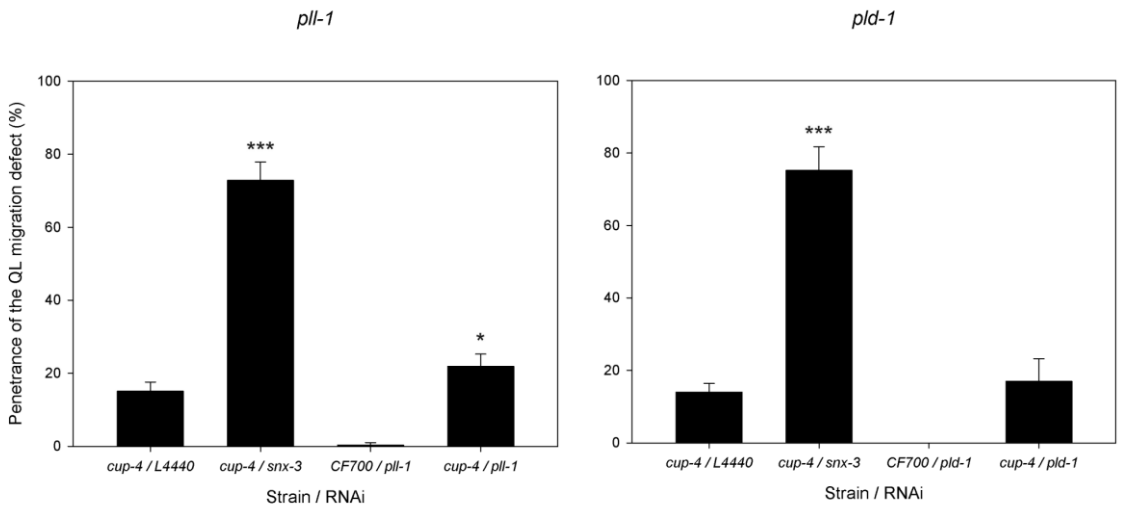

Figure 11: Phospholipase C-like and phospholipase D

Genetic interactions of *cup-4* with phospholipase C-like (*pll-1*) and phospholipase D (*pld-1*) as determined by RNAi on *cup-4 (ok837); muIs32* and CF700 (*muIs32*) strains. *pll-1* RNAi caused slight enhancement of the defect in QL migration of *cup-4* mutants which was significant on 5% level, while RNAi against *pld-1* had no significant effect.

For both genes, RNAi against *snx-3* is also shown as a positive control. Bars represent mean values of three independent experiments (n = 3), each containing usually from 90 to 100 animals (N = 90 – 110); error bars represent standard deviations. Since all genes could not always be tested in the same batch, controls are slightly different for each gene; that is why separate bar plot with separate controls are shown for each gene.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, unpaired *t*-test (compared to L4440 RNAi on *cup-4 (ok837); muIs32* strain)

Table 4 **Phospholipases***

Gene	Phospolipase homologue	Relevant phenotype
plc-1	C	Interaction with β-catenin BAR-1
plc-2	C(-beta?)	?
plc-3	C-gamma	Receptor mediated endocytosis defective
plc-4	C(-delta)	?
pll-1	C-like	?
egl-8	C-beta	Egl phenotype as in the absence of Wnt EGL-20
pld-1	D	Maturation of phagosomes
ipla-1	DDHD1	Involved in endosome-to-Golgi retrograde trafficking

* source: http://www.wormbase.org

5.2.6 Phosphatidylinositol 3-kinases *vps-34* and *age-1*

Phosphatidylinositol 3-kinase VPS-34 counteracts the action of myotubularin phosphatidylinositol 3-phosphatases in regulation of PI-3 level on early endosomes (Silhankova et al., 2010). It can hence regulate binding of retromer and consequently, recycling of Wntless to allow secretion of Wnt and represent another potential player in Wnt secretion and signalling.

Another phosphatidylinositol 3-kinase, AGE-1, also acts on the third position, but in contrast to VPS-34, which uses phosphatidylinositol as a substrate, AGE-1 phosphorylate PI-4,5-P_2 to PI-3,4,5-P_3. This opens the possibility, that AGE-1 could be responsible for diminishing of PI-4,5-P_3 in *cup-4* mutants by converting it to PI-3,4,5-P_3. If this would be the case, I would expect, that knockdown of AGE-1 could rescue *cup-4* QL migration defect. This, of course, depends on the premise, that this phenotype is caused by deregulated levels of PI-4,5-P_2 or PI-3,4,5-P_3. PI-4,5-P_2 has been explicitely demonstrated to play a role in signal transduction in receiving cells (Pan et al., 2008b; Tanneberger et al., 2011) and it is likely to be involved in the endocytosis of Wls in Wnt producing cells as well. But if shifted balance of phosphoinositides would mean that *cup-4* mutation causes general deregulation of (3-)phosphoinositide kinases, then also VPS-34 would be affected. VPS-34 regulates pool of phosphoinositides shown to be essential for Wnt signalling; this is why *vps-34* RNAi was also performed.

In spite of that, I found no effect on the QL migration defect when either genes where knocked down on wt or *cup-4* mutant background (Figure 12). However, the reduction of function caused by RNAi does not have to be sufficient to cause the visible effect, yet complete elimination of function could cause QL migration defect. Thus, it still cannot be ruled out that deregulation of *age-1* and/or *vps-34* PI-3 kinases is a cause of *cup-4* QL migration defect. Moreover, the hypothesis that the decreased PI-4,5-P2 levels of *cup-4* mutants and hence their endocytic and possibly also Wnt defects are the results of the hypoactivation of PI 5-kinase was not tested yet.

5.3 Rescue assay

To find out, in which cells CUP-4 function is required for Wnt signalling, we decided to perform rescue assay with *cup-4* expressed in *cup-4 (ok837)* mutant background from different tissue specific promoters, active only in coelomocytes, EGL-

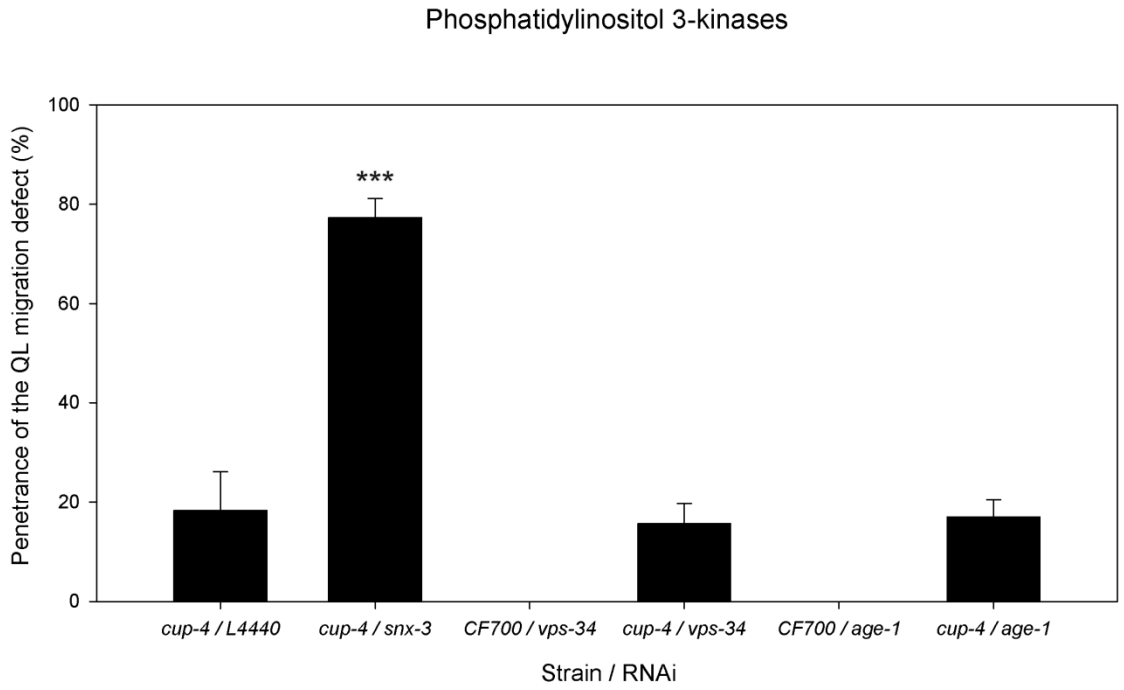

Figure 12: Genetic interaction between *cup-4* and PI 3-kinases

Results of RNAi against *vps-34* and *age-1* phosphoinositide 3-kinases on wild type or *cup-4* mutant strain. No additional enhancement of the QL migration defect could be seen in either *vps-34* or *age-1* when knocked down on the *cup-4* mutant background, nor did any of these genes caused the QL migration defect on the WT background. For both genes, RNAi against *snx-3* is also shown as a positive control. Bars represent mean values of three independent experiments (n = 3), each containing 100 animals (N = 10); error bars represent standard deviations.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, unpaired *t*-test (compared to L4440 RNAi on *cup-4 (ok837); muIs32* strain)

20/Wnt producing or receiving cells, respectively. Rescue assay was previously used several times to prove specific function of proteins participating on Wnt signalling in Wnt producing cells of *C. elegans* (Coudreuse et al., 2006; Silhankova et al., 2010).

I first prepared a construct with *cup-4* expressed under *egl-20* promoter. To monitor CUP-4 expression, CUP-4 encoded by this construct was marked by GFP inserted inside the protein. Such recombinant protein was already previously used and proved to be functional (Patton et al., 2005). However, using either wide field or confocal fluorescence microscopy, I was not able to detect any GFP in transgenic animals, although they strongly expressed co-injection marker in their pharynxes (Figure 13). One possible explanation is that not enough plasmid construct DNA was injected into worms‘ gonads and using a higher input material could lead to positive result in the future.

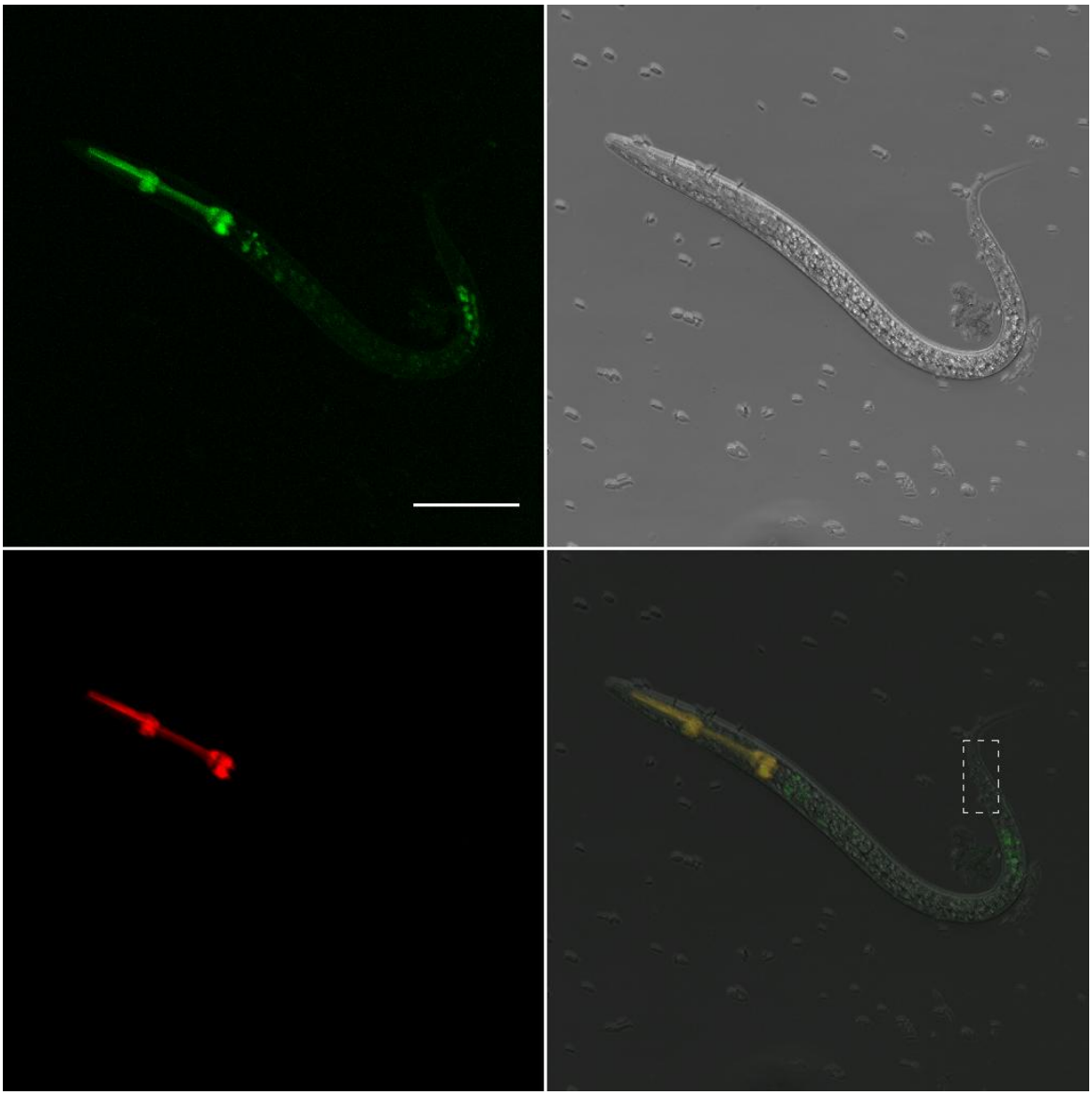

Figure 13: Transgenic animals

pPD49.26 plasmid carrying *egl-20p::cup-4::gfp* construct was injected into gonads of *cup-4 (ok837)* hermaphrodites along with coinjection marker *[Pmyo-2::tdTomato]*. Although marker was expressed in the pharynx of transgenic animals, even with confocal fluorescence microscope, we were unable to detect any CUP-4::GFP (green signal comes from autofluorescence of intestinal granules located anterior to EGL-20/Wnt producing cells as can be seen in overlay image, where EGL-20/Wnt cells are demarkated by dashed box. tdTomato in pharynx is also emmiting in the green channel.

Scale bar equals 50 µm and applies for all images. Green, red and bright field channel with overlay of one representative specimen are shown. Images are maximal projections of eight subsequent sections taken with *z* steps of 0,49 µm.

5.4 Does *cup-4* mutation affect MIG-14/Wls levels and localization?

To further address the question, if CUP-4 is somehow involved in recycling MIG-14/Wls from the plasma membrane, I crossed *huSi2* single copy transgene (expressing MIG-14::GFP fusion protein from a *mig-14* promoter) in *cup-4 (ok837)* mutant background (see chapter Methods, section 4.3.3.1 for details of crossing).

With this strain, I performed two experiments to compare changes (if any) in MIG-14::GFP protein levels and localization. First, I did Western blots with anti-GFP antibody. Unfortunately, in my hands, with protocols I tried and with antibody batch I used, this was not working well and I was not able to detect MIG-14::GFP (see Methods, chapter 4.8 for details) although I detected both tubulin and GFP secreted into the body cavity of *arIs37; dpy-20* strain (data not shown).

Secondly, I investigated the level and localization of MIG-14::GFP in EGL-20 producing cells of wild type, *cup-4 (ok837)* mutant strains using confocal fluorescence microscopy. I also included *mtm-6 (ok330);huSi2* strain in the analysis for comparison.

Mutation in *mtm*-6 gene encoding myotubularin phosphoinositide 3-phosphatase leads to a partial decrease in MIG-14::GFP fusion protein levels (Silhankova et al., 2010).

Although no apparent decrease in MIG-14::GFP can be seen, the pattern of MIG-14/Wls in *cup-4 (ok837); huSi2* appears to be more punctate (Figure 14). Homogenous plasma membrane localization, which can be observed between these punctae in wild type worms is no longer apparent. This could be also a consequence of overall decrease of MIG-14::GFP level. Also the background noise is higher than in *huSi2* control, suggesting that overall signal is lower in *cup-4 (ok837)* mutants. Taken together, these observations lead to conclusion that although MIG-14/Wls::GFP protein levels might be mildly lowered, when CUP-4 function is missing, this reduction is not even remotely as apparent as in *mtm-6 (ok330)* mutants, where MIG-14/Wls::GFP protein levels are visibly reduced. This is in agreement with the observation, that QL-migration defect of *cup-4* mutants also has lower penetrance than that of *mtm-6* mutants, indicating that more MIG-14/Wls is retained in *cup-4* (if we accept the idea that alterations of MIG-14/Wls levels available for EGL-20/Wnt secretion are indeed the cause of *cup-4* mutant phenotype).

Figure 14: MIG-14 levels and localization on wt, *cup-4* and *mtm-6* mutant background (see next page):

Confocal fluorescence microscopy images of tails of *C. elegans* L1 larvae carrying *huSi2* transgene on wild-type, *cup-4 (ok837)* or *mtm-6 (ok330)* mutant background, respectively. *huSi2* is a single copy integrated stable transgene encoding MIG14/Wntless labeled with GFP (MIG-14::GFP) driven by *mig-14* endogenous promoter. Thus, its expression is likely to reflect the real situation *in vivo.* Larvae were synchronized by washing plates with laid embryos by M9 buffer. Embryos that remained on the plate were allowed to hatch and feed on OP50 *E. coli* bacteria for 4,5 hours before plates were washed again, worms pelleted down, mounted to a microscopic slide on an agarose pad containing 10mM sodium azide and used for microscopy. Note the group of EGL-20/Wnt producing cells around the distal intestine (rectum, opening ventrally). Highest accumulation of MIG-14/Wls can be seen on the luminal side of the cells. Signal in the very end of the tail marks hypodermal cells expressing LIN-44/Wnt. Red channel shows autofluorescence of intestinal granules, which emits in both green and red channels, and in merge allows to distinguish them from MIG-14::GFP signal.

The pattern of MIG-14/Wls in *cup-4 (ok837); huSi2* appears to be more punctate with lack of visible membrane fraction compared to wild type strain. Also the background noise is higher than in *huSi2* control, suggesting that overall signal is lower in *cup-4 (ok837)* mutants. Taken together, these observations lead to conclusion that although MIG-14/Wls::GFP protein levels might be mildly lowered, when CUP-4 function is missing, this reduction is not even remotely as apparent as in *mtm-6 (ok330)* mutants, where MIG-14/Wls::GFP protein levels are highly reduced.

In all images anterior is left, dorsal is up. The individuals shown are representative specimens chosen from at least 10 photo sets (except for *mtm-6 (ok330); huSi2*, where *n* was 5) and many other observations made for each genotype. Images are maximal projections of 10 subsequent photographs in *z* taken with *z*-steps of 0,326 µm. Scale bar represents 20µm and applies for all images. Setting of the microscope was the same for all images. Maximal projections were made in FIJI software. False colours were assigned to channels using the same software and images were converted from 12-bit to the RGB format; otherwise no modifications of original photographs were made.

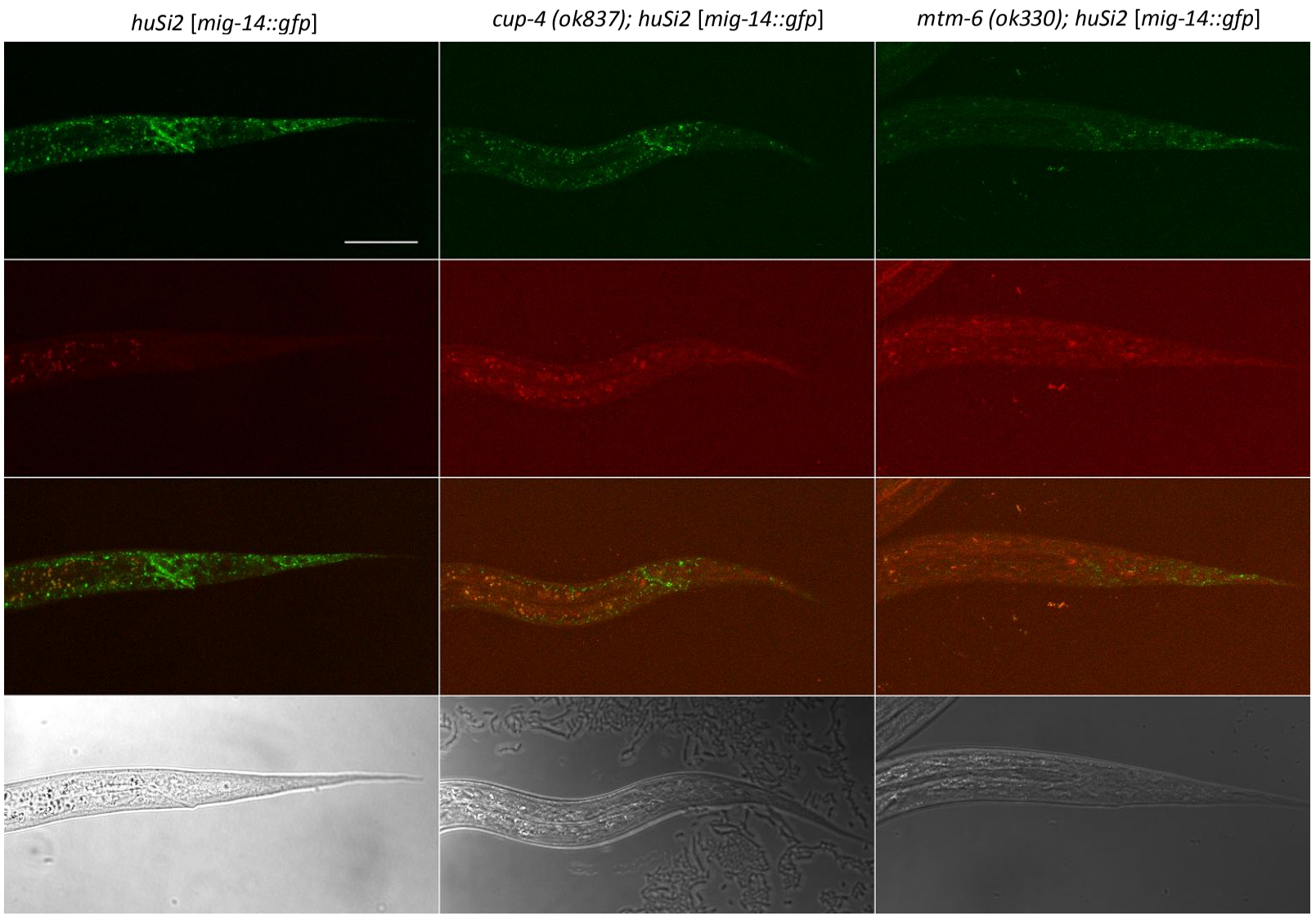

Figure 14: MIG-14 levels and localization on wt, *cup-4* and *mtm-6* mutant background – for description see the previous page

6 Discussion

6.1 Placement of CUP-4 into the Wnt signalling

We planned to determine the site of CUP-4 action in the Wnt signalling by performing the rescue assay, i. e. by expressing wild type *cup-4* in the *cup-4 (ok837)* mutant background under various tissue specific promoters. However, this experiment was unsuccesful – besides I encountered many difficulties during cloning, the construct, when finally prepared and injected, seemed not to be expressed. Thus, I have to rely on indirect evidence from my other experiments and informations from literature to at least try to place CUP-4 into the Wnt signalling pathway.

First, I investigated the genetic interaction of *cup-4* and *pry-1*, the gene encoding a *C. elegans* homologue of Axin (Korswagen et al., 2002). PRY-1/Axin is a component of the destruction complex which in canonical Wnt signal transduction pathway targets β-catenin in signal receiving cells for degradation (Kikuchi et al., 2009). I found out that both *pry-1 (mu38); cup-4 (ok837)* double mutant and *pry-1 (mu38)* single mutant animals exhibit highly penetrant defect in the migration of QR but not the QL. These findings testify that even when *cup-4* mutation is present, Wnt signalling is still ectopically activated by elimination of PRY-1 function. This leads to the conclusion that CUP-4 acts upstream of PRY-1 so when Wnt signalling is activated by the elimination of PRY-1/Axin, *cup-4* mutation cannot manifest itself. The deficiency caused by mutation in *cup-4* would be then fully compensated by downstream Wnt-independent stabilization of β-catenin caused by the lack of functional PRY-1 protein. Conversely, if CUP-4 would function downstream of PRY-1, I would rather expect that *cup-4 (ok837)* would at least partially compensate for the ectopic activation of Wnt pathway caused by *pry-1* mutation.

Instead, the penetrance of the QR migration defect of *cup-4; pry-1* double mutant was a little bit higher than that of *pry-1* single mutant. The cause of this difference is so far unknown. Migration of the QR neuroblast is regulated by multiple Wnts, which can function as both repellents or attractants (Zinovyeva et al., 2008). Although the QR neuroblast is turned to the QL-like fate by *pry-1* mutation, this reversion may not be absolute. Precise positions of both QR and QL could be still regulated by other Wnts, which may function through other pathways that do not require PRY-1. I thus speculate that signalling of these other Wnts can be compromised in *cup-4* mutants leading to more QR neuroblasts remaining more posteriorly and hence to more penetrant QR migration

defect. However, none of the strains displays absolutely penetrant defect in the QR migration. I argue that this is because of persisting function of another Axin homologue, AXL-1 (Oosterveen et al., 2007).

Another evidence comes from knock down of *apa-2* and *dpy-23*, components of AP2 adaptor complex for clathrin mediated endocytosis. When AP2 function is eliminated, MIG-14, the *C. elegans* homologue of Wnt cargo receptor Wntless, cannot be endocytosed and accumulates on the membranes of Wnt producing cells. The same phenotype can be observed when RNAi is performed on *cup-4 (ok837)* mutant background. Furthermore, *cup-4* mutant phenotype itself is similar to wild type and different from AP2 RNAi phenotype – MIG-14 is distributed in punctae and it is not trapped on the plasma membrane. This suggests that *cup-4* mutation does not interfere with transport of Wntless to the plasma membrane, because in *cup-4* mutant animals exposed to RNAi against AP2 subunits, MIG-14 still can reach the membrane and accumulate there. At the same time, MIG-14 is not trapped on the cell surface in *cup-4* mutants themselves concluding that CUP-4 does not function in the same way as AP2. It can either function anywhere downstream of AP2 or affect the Wnt protein processing but not Wntless trafficking before Wnt-Wntless complex reaches the plasma membrane during secretion. Since the passage of Wnt (and hence Wls) through the secretion route often depends on the proper folding and modifications of Wnt protein, Wls role in these processes also seems unlikely. Beacause one cannot say from confocal images where exactly is the MIG-14 localized in *cup-4* mutants, it is still possible that MIG-14 is in fact trapped on the cytoplasmic membrane when CUP-4 function is lacking, but it forms the accumulations instead of being homogenously distributed as when AP2 is depleted.

These results narrow the original range of potential roles of CUP-4 in Wnt signalling but do not allow to discriminate between the three hypothesis regarding the place of CUP-4 action in Wnt signalling delineated in chapter 3. Yet, we favour the first option (CUP-4 functions in EGL-20/Wnt producing cells) because of the following reasons:

First, CUP-4 is known to be essential for endocytosis and the importance of endocytosis for retrieving of Wntless from plasma membrane of Wnt secreting cells has been clearly demonstrated (Pan et al., 2008a; Port et al., 2008; Yang et al., 2008). Moreover, other Cup mutants discovered to display Wnt-related defects, *cup-6* and *cup-10* (later homologized to myotubularin genes and renamed to *mtm-6* and *mtm-9*) (Dang et al., 2004), were shown to be specifically required for Wntless recycling in Wnt producing

cells (Silhankova et al., 2010). It is probable that if they function together in one process (endocytosis by coelomocyte) they also function toghether in the other (Wnt signalling).

Secondly, a very strong genetic interactions between *cup-4* and *mtm-6* or *snx-3* plus a little bit weaker but still strong interaction with *vps-29* suggest involvement of their protein products in the same processes. Although this process can be considered to be the whole Wnt signalling, it is more probable, that they are more closely related. Since all *mtm-6*, *vps-29* and *snx-3* act in the signal producing cell to ensure Wls recycling (Coudreuse et al., 2006; Harterink et al., 2011b; Silhankova et al., 2010) CUP-4 is likely to function in the same cell.

Third, although not prominent or very convincing, MIG-14/Wls seemed to be present in somewhat lower levels in *cup-4* mutants, although this needs to be confirmed by more accurate methods like Western blotting.

On the other hand, endocytosis, which is now the main mechanism known to be regulated by CUP-4, plays also a role in Wnt signal receiving cells but whether this role is positive or negative is still a subject of disputations (Gagliardi et al., 2008). Unfortunately no data addressing the role of endocytosis in receiving cells are available in *C. elegans*.

The indirect effect of *cup-4* mutation on Wnt signalling, no matter how unlikely, still cannot be ruled out. Few pieces of evidence even point to this direction and show the way, how this could be achieved. When *cup-4* expression was examined, it was found to be specifically expressed in coelomocytes but not reported in other cells, including EGL-20 producing cells or Q neuroblasts (Patton et al., 2005).

6.2 Possible mechanisms of CUP-4 function

Answer to the question of how CUP-4 could affect Wnt signalling is dependent on its place of function only to some extent. I already mentioned in the previous section of this chapter that no matter where the action of CUP-4 takes place, the *cup-4* Wnt phenotype could be caused by the observed endocytic defect. The whole problem then reduces to how *cup-4* mutation disrupts endocytosis.

In signal receiving cells, endocytosis could be required for internalization of Wnt-Frizzled complexes, LRP5/6 (summarized in (Gagliardi et al., 2008)), stabilization of Dishevelled (Bryja et al., 2007) or for sequestration of GSK-3β into multivesicular endosomes and thus protecting β-catenin from degradation (Taelman et al., 2010).

The event requiring endocytosis in Wnt producing cell is the internalization of Wls after it has delivered Wnt to the cell surface so it could be recycled back to the TGN for repeated use (Pan et al., 2008a; Port et al., 2008; Yang et al., 2008). Retromer acts downstream of the endocytic step to ensure retrieval of Wls from endosomes (Belenkaya et al., 2008; Franch-Marro et al., 2008), an event tightly regulated by levels of 3-phosphoinositides (Harterink et al., 2011b; Silhankova et al., 2010).

This is why I investigated the genetic interactions of *cup-4 (ok837)* with components of retromer. The non-viability of *cup-4 (ok837); vps-29 (tm1320); muIs32* homozygotes / heterozygotes suggests strong genetic interaction. On the other hand, it is not clear whether this can be assigned to the disruption of Wnt signalling or some other functions of CUP-4 and VPS-29. Nevertheless, I later confirmed the interaction of both genes in EGL-20/Wnt signalling by performing RNAi against *vps-29* on *cup-4* mutant background.

I did not find any effect of *vps-35* RNAi on the penetrance of QL migration defect in *cup-4* mutant worms nor I observed a QL mutant phenotype in *vps-35* knock down on wild type strain, although this was reported to be very strong (Coudreuse et al., 2006). I thus argue that the knock down in this particular case was not succesful.

Very strong interaction was uncovered by RNAi between *cup-4 (ok837)* and *snx-3*, a gene which encodes a sorting nexin. SNX-3 recruits cargo-selective subcomplex of retromer and Wntless to budding endosomal vesicles containing 3-phosphoinositides (Harterink et al., 2011b). Similarly strong interaction was also found between *cup-4* and *mtm-6*. MTM-6 is a phosphatase that dephosphorylates 3-phosphoinositides, thus regulating their levels and membrane association of SNX-3 (Silhankova et al., 2010) which bind to 3-phosphoinositides by its PX domain (Harterink et al., 2011b). Elimination of either *snx-3* or *mtm-6* function leads to a strong enhancement of the *cup-4 (ok837)* migration defect. Moreover, the effect in all *vps-29, snx-3* and *mtm-6* is rather synthetic than simply additive, i. e. the double mutants (or mutant/RNAi) display more severe defect (expressed as a penetrance of the phenotype) then the sum of the phenotype penetrance from both single mutants combined (or mutant/no RNAi + wt/RNAi). Also MIG-14::GFP, which is observed in severely reduced levels in *mtm-6* mutants likely due to shifted balance of 3-phosphoinositid content in endosomal membranes and thus impaired retrieval and increased lysosomal degradation (Silhankova et al., 2010), seems to be present in somewhat lower levels in *cup-4* mutans, although this decrease is not as apparent and needs to be confirmed by other experiments.These observations lead

to the conclusion that CUP-4 functions in the same process as VPS-29, SNX-3 and MTM-6, although it cannot distinguish between function in the same or parallel pathways nor it can completely rule out the possibility that this process is Wnt signalling as a whole.

One of the most apparent effect of *cup-4* loss of function in coelomocytes is the drastically decreased level of phosphatidylinositol-4,5-bisphosphate. It was speculated, that this is caused by the hyperactivation of phospholipase C, which use PI-4,5-P_2 as a substrate (Patton et al., 2005). PI-4,5-P_2 produced in the receiving cells in response to Wnt signal (Pan et al., 2008b) is required to bring scaffold protein Amer1 to cytoplasmic membrane. Amer1 recruits GSK-3β and CK-Iγ and thus enhances LRP-5/6 phosphorylation and stimulates Wnt/β-catenin signalling (Tanneberger et al., 2011). Disruption of this process represents another very plausible explanation of Wnt defects observed in *cup-4* mutants. Decrease in PI-4,5-P_2 levels would then cause endocytic defect in parallel to Wnt signalling defects.

Straight connection between this phenotype, endocytosis and Wnt signalling exists, since AP2, an adaptin required for clathrin-dependent endocytosis of MIG-14/Wls for its recycling and repeated use (Pan et al., 2008a; Yang et al., 2008), is brought to the membrane by binding to PI-4,5-P_2 (Höning et al., 2005). However, I made the observation that in *cup-4* mutants, GFP-tagged MIG-14/Wls is localized in a punctate pattern which is distinct from that of RNAi against AP2 subunits, where MIG-14::GFP is trapped and homogenously distributed on the membranes. This argue against the possibility that the Wnt signalling defect of *cup-4* mutants is caused by the decrease of PI-4,5-P_2 levels which, in turn, impedes binding of AP2 to the cytoplasmic membrane and internalization of Wls in Wnt producing cells. On the other hand, *cup-4* Wnt phenotype is of the low penetrance and so could be the defect in AP2 binding which could therefore escape from detection. Yet, together with the role of PI-4,5-P_2 on LRP-5/6 phosphorylation in signal receiving cells, disruption on AP2 binding to membranes due to decreased PI-4,5-P_2 levels and thus impaired Wls recycling remains the most plausible explanation of *cup-4* Wnt-related defects.

It is also possible that the lack of CUP-4 function leads to a general deregulation of the phospholipase or PI kinase function, which leads to both decreased level of PI-4,5-P2 and the disbalance in the levels of 3-phosphoinositides. PI-3-P regulates binding of the retromer subunit SNX-3 to endosomal membranes. In turn, SNX-3 recruits cargo-selective subunit of retromer and Wls into budding vesicles which can then be retrieved back to the TGN. Deregulation of phospholipases or PI kinases could be achieved directly

of indirectly. For example, the small GTPase Arf6 activites the PI 5-kinase which synthesise PI-4,5-P_2 from PI-4-P (Brown et al., 2001) and at the same time also operates in the same pathway and partially colocalize with MTM-6/-9 myotubularins (Dang et al., 2004) which in turn regulate Wls recycling and hence Wnt signalling (Silhankova et al., 2010). It is then easy to imagine, that if CUP-4 would be somehow required for activation of ARF-6, its missing function would simultaneously lead to decreased PI-4,5-P_2 levels and disruption of Wnt signalling. Altered phosphoinositide levels (namely diminished PI-4,5-P_2) would then be not a cause, but a consequence or a side effect of the event leading also to disruption of Wnt signalling.

I thus performed RNAi against all available phospholipases C and two phosphoinositide 3-kinases that could be responsible for *cup-4* mutant phenotypes. I would expect a rescue of the phenotype when the phospholipase hyperactivated in *cup-4* mutant would be eliminated by RNAi. However, I found no effect in PI 3-kinases and most phospholipases. Only knock down of *pll-1* encoding phospholipase C-like protein, displayed weakly significant enhancement of *cup-4* mutant phenotype. However, as mentioned earlier, if the cause of *cup-4* QL migration defect would be indeed upregulation of phospholipase activity, I would expect rather rescue than enhancement of the phenotype.

Although involvement of phospholipases and phospatidylinositol 3-kinases on Wnt signalling seemes to be – based on my findings – unlikely, the interpretation of the results is tricky. Not only that several phospholipases can act redundantly in regulation of phosphoinositide levels, but also (and this applies for all proteins tested) the absence of effect can also mean that the investigated protein and CUP-4 function in the same process, which is already completely disrupted by *cup-4* mutation but is not absolutely indispensable for Wnt signalling (hence we do not see completely penetrant phenotype).

Other reason of incomplete penetrance of *cup-4* mutant phenotype could be the retention of some residual activity by mutant CUP-4, which is quite unlikely because *cup-4 (ok837)* is an extensive frame shift deletion mutation which eliminates most of the protein (source: *http://www.wormbase.org*). Existence of a redundantly acting related protein cannot be ruled out, since nicotinic acetylcholine receptor gene family where *cup-4* belongs has at least 54 members in *C. elegans* (Jones and Sattelle, 2004). However, *lgc-26*, whose mutant displays same defect in coelomocyte endocytosis as *cup-4* seems to encode protein that functions rather together in one complex with CUP-4

than redunduntly with it, since neither of the two genes can complement for mutation of the other (Patton et al., 2005).

There is another option how activation of phospholipase C could affect Wnt signalling without including 3-phosphoinositides but explaning decreased levels of PI-4,5-P_2:

Phospholipase C cleaves the second messenger inositol-1,4,5-trisphosphate from PI-4,5-P_2. IP_3 is known to open ion channels in the ER and release Ca^{2+} which regulates endocytosis via activation of the protein phosphatase calcineurin. Ca^{2+}-calcineurin dephosphorylates and inactivates GTPase dynamin. Dynamin is necessary for cleaving off the budding endocytic vesicles from the membrane. Such relationship was shown also for *C. elegans* dynamin homologue DYN-1 in a pathway that comprises also CUP-4 (Song et al., 2010). The importance of endocytosis in Wnt signalling has been already discussed. If hyperactivated, phospholipase C would deplete PI-4,5-P2 which levels would thus decline. Elevated concentration of Ca^{2+} activates calcineurin which would in turn inactivate dynamin and thus prevent endocytosis and Wnt signalling. This scenario accounts for all *cup-4* defects: the decreased PI-4,5-P_2 level and through the disruption of endocytosis to the defect in Wnt signalling.

Another possibility is that the Wnt signalling is compromised because of the lack of CUP-4 ion channel function and hence disbalance in ion concentrations. For example, non-canonical Ca^{2+} signal transduction pathway in receiving cells could act in parallel as a minor pathway to the main canonical β-catenin pathway (Kikuchi et al., 2009). It could require CUP-4 to release Ca^{2+} and when eliminated, account for observed low-penetrant QL migration defect of *cup-4* mutants.

Expression pattern of CUP-4 as published in (Patton et al., 2005), where CUP-4 was found to be expressed only in coelomocytes, suggests that CUP-4 should affect Wnt signal not in the Wnt producing or receiving cells, but on the way between them by CUP-4 role in coelomocytes. Although low amounts of reporter protein could be overlooked or the sequence used to drive its expression may not encompassed the whole *cup-4* promoter, let's for a moment assume, that the expression was described correctly and CUP-4 can be indeed found only in coelomocytes. How could it then affect Wnt signalling?

Coelomocytes non-specifically endocytose fluid from the pseudocoelom (Fares and Greenwald, 2001). Although transcellular transport and thus a need for endocytosis has also been suggested (Bejsovec and Wieschaus, 1995), Wnt proteins are generally

considered to spread in extracellular space and hence could be endocytosed by coelomocytes as well. If this would be the case, I would expect the reversed effect than we actually see in *cup-4* mutants, i. e. in mutants with prevented endocytosis of Wnt by coelomocytes, more Wnt should be present in extracellular space and the signalling should be enhanced instead of being compromised. More interesting opportunity opens with secreted Frizzled receptor SFRP-1, which anteriorly cuts-off Wnt gradients including that of EGL-20 (Harterink et al., 2011a). If SFRP-1 would be partially cleared from body of wild type worms by functional coelomocyte endocytosis, this would ensure enough EGL-20/Wnt to be free to reach Q neuroblasts, which are located quite distantly to anterior from the EGL-20 source. When the endocytosis by coelomocytes is disrupted, e. g. by *cup-4* mutation, more SFRP-1 would then remain in the body cavity and bind most of EGL-20. Concentration of free EGL-20 would be below the threshold able to activate signalling cascade in Q neuroblasts. The only problems of this scenario are that it is purely speculative and it would demand specific internalization of SFRP-1, whereas endocytosis of coelomocytes is referred to be non-specific (Fares and Greenwald, 2001) (which doesn't mean they do not have receptor mediated endocytosis – in fact, they probably have). Otherwise, simultaneous non-specific endocytosis of SFRP-1 and EGL-20 would have probably no net effect on signalling.

Here, I narrowed the range of where CUP-4 could function in Wnt signalling and presented several scenarios of how elimination of CUP-4 function could lead to observed phenotypes. We favoured the one where the Wnt phenotype would be a result of hyperactivation of phospholipase C, decreased levels of PI-4,5-P2 and impaired binding of AP2 adaptin which would prevented efficient Wls recycling and Wnt secretion. However, my results bring low support to this hypothesis, yet, they do not completely rule it out nor they are conclusive enough to discriminate between the others. I found out that CUP-4 in Wnt signalling functions upstream of PRY-1/Axin and most probably downstream of AP2. It is involved in the same process as MTM-6, SNX-3 and VPS-29, which takes place in the Wnt producing cells, yet, it still cannot be ruled out that this process is the whole Wnt signalling itself.

7 Conclusions

CUP-4 is a protein previously showed to be important for efficient endocytosis by coelomocytes (Fares and Greenwald, 2001; Patton et al., 2005), involved in dietary restriction induced longevity (Park et al., 2010), oxidative stress resistance and maintaining of normal life span (Park et al., 2009). It has also been demonstrated to be required for fully functional EGL-20/Wnt signalling that guide migrations of the Q neuroblasts during *Caenorhabditis elegans* development (Silhankova et al., 2010).

Here, I show that *cup-4; pry-1*double mutants exhibit *pry-1* phenotype and hence CUP-4 functions upstream of PRY-1/Axin. Similarly, *dpy-23* or *apa-2* knockdown leads to accumulation of MIG-14/Wls on the cell surface on both wild type and *cup-4* mutant background. CUP-4 thus most probably acts downstream of AP2 clathrin adaptor complex or is not involved in MIG-14 cycle in EGL-20/Wnt producing cells at all. Since the main experiment which was supposed to place the CUP-4 action in Wnt signalling into signal producing or receiving cell turned out not to work, I managed only to narrow the possible site of action of CUP-4 in Wnt signalling. Yet, indirect evidence of data from literature and my own experiments slightly favour the placement of CUP-4 function in Wnt signalling into Wnt producing cells.

I found that mutation of *cup-4* strongly genetically interacts with genes encoding components of retromer, *vps-29* and *snx-3*. On the other hand, no interaction with *vps-35* was found, but this was probably due to an inefficient knock down. Very strong genetic interaction was also proven between *cup-4* and *mtm-6*, a gene encoding myotubularin phosphoinositide 3-phosphatase. All VPS-29, SNX-3 and MTM-6 regulate recycling of MIG-14/Wls, a Wnt cargo receptor in EGL-20/Wnt producing cells (Harterink et al., 2011b; Silhankova et al., 2010; Yang et al., 2008).

I critically analyzed the informations available in the literature and together with my data, I delineated several possible explanations of where and how CUP-4 could participate on Wnt signalling. Using knockdown by RNAi, I checked genetic interactions between *cup-4* mutant allele and several genes whose products were implicated to function it these scenarios, namely phospholipases C and PI-3 kinases. I found no strong genetic interaction between *cup-4* and any of these genes nor knock down of any of them caused the defect in EGL-20/Wnt signalling on their own. In phospholipases, I did not find the rescue of the *cup-4* QL phenotype arguing against the favoured

hypothesis in which the Wnt-related defect should be caused by hyperactivation of phospholipase C. Also localization of GFP-tagged MIG-14/Wls in *cup-4* mutants does not support the role of CUP-4 in maintaining PI-4,5-P_2 levels, thus recruiting of AP and regulating Wls recycling. I also did not find any genetic interaction between *cup-4* and *vps-34* despite VPS-34 PI 3-kinase was shown to regulate Wls recycling (Silhankova et al., 2010).

Another observation has been made on MIG-14::GFP, whose levels might be somewhat reduced on *cup-4* mutant background. Together with known importance of endocytosis for secretion of Wnt these findings suggest possible effect of *cup-4* mutation on Wntless recycling in Wnt producing cells. However, the evidence is not convincing and Western blots to determine precisely changes in MIG-14/Wls levels also did not work properly.

With my work on this thesis, I prepared ground for future research on this topic and delineated its possible future ways. For example, it would be interesting, if *lgc-26* mutation would cause the same QL migration defect as *cup-4* and if it could enhance its penetrance or if PI 5-kinase or ARF-6 are somehow involved in Wnt signalling. Although we learned something about CUP-4 interactions and function, clearly, more experiments, for example successful determination of the tissue specificity of CUP-4 function in Wnt signalling by the rescue assay and following colocalization studies or precise determination of changes in MIG-14 levels on Western blots will be necessary to unambiguously elucidate the exact role and place of CUP-4 in Wnt signalling.

8 Acknowledgement

I thank to my parents for their endless patience and unbelievable support during writing the thesis and through all my previous studies – nothing of this would be possible without them.

I thank to my supervisor, Mgr. Marie Macůrková, Ph.D., for her valuable comments on the thesis, advices and help in the laboratory, her husband, MUDr. Libor Macůrek, Ph.D., for help with preparation of the *egl-20p::cup-4::gfp* construct and MSc. Masako Asahina-Jindrová, Ph.D. for injecting the constructs into worms. I also thank to all the other members of our laboratory for occasional help, especially to our technician, Bc. Jitka Velčevová for answering my questions regarding preparation of various solutions, chemicals and worm cultivation and also for providing me some six-well plates for RNAi. Last, but not least, I would like to thank to Ing. RNDr. Vladimír Krylov, Ph.D., RNDr. Lenka Libusová, Ph.D. and Doc. RNDr. František Půta, Csc. for consultations and to Mgr. Ondřej Šebesta for help with confocal microscope settings.

This work was financed from GAČR grant P305/10/0507 to Mgr. Marie Macůrková, Ph.D.

9 Literature

Arighi, C. N., Hartnell, L. M., Aguilar, R. C., Haft, C. R. and Bonifacino, J. S. (2004). Role of the mammalian retromer in sorting of the cation-independent mannose 6-phosphate receptor. *Journal of Cell Biology* **165**, 123-133.

Baeg, G. H., Selva, E. M., Goodman, R. M., Dasgupta, R. and Perrimon, N. (2004). The wingless morphogen gradient is established by the cooperative action of Frizzled and Heparan Sulfate Proteoglycan receptors. *Developmental Biology* **276**, 89-100.

Banziger, C., Soldini, D., Schutt, C., Zipperlen, P., Hausmann, G. and Basler, K. (2006). Wntless, a conserved membrane protein dedicated to the secretion of Wnt proteins from signaling cells. *Cell* **125**, 509-522.

Bartscherer, K., Pelte, N., Ingelfinger, D. and Boutros, M. (2006). Secretion of Wnt Ligands requires Evi, a conserved transmembrane protein. *Cell* **125**, 523-533.

Bejsovec, A. and Wieschaus, E. (1995). Signaling activities of the Drosophila wingless gene are separately mutable and appear to be transduced at the cell surface. *Genetics* **139**, 309-20.

Belenkaya, T. Y., Wu, Y. H., Tang, X. F., Zhou, B., Cheng, L. Q., Sharma, Y. V., Yan, D., Selva, E. M. and Lin, X. H. (2008). The retromer complex influences Wnt secretion by recycling Wntless from endosomes to the trans-Golgi network. *Developmental Cell* **14**, 120-131.

Brown, F. D., Rozelle, A. L., Yin, H. L., Balla, T. and Donaldson, J. G. (2001). Phosphatidylinositol 4,5-bisphosphate and Arf6-regulated membrane traffic. *Journal of Cell Biology* **154**, 1007-1017.

Bryja, V., Cajanek, L., Grahn, A. and Schulte, G. (2007). Inhibition of endocytosis blocks Wnt signalling to β-catenin by promoting dishevelled degradation. *Acta Physiologica* **190**, 55-61.

Carlton, J., Bujny, M., Peter, B. J., Oorschot, V. M. J., Rutherford, A., Mellor, H., Klumperman, J., McMahon, H. T. and Cullen, P. J. (2004). Sorting nexin-1 mediates tubular endosome-to-TGN transport through coincidence sensing of high-curvature membranes and 3-phosphoinositides. *Current Biology* **14**, 1791-1800.

Clevers, H. (2006). Wnt/beta-catenin signaling in development and disease. *Cell* **127**, 469-480.

Coombs, G. S., Yu, J., Canning, C. A., Veltri, C. A., Covey, T. M., Cheong, J. K., Utomo, V., Banerjee, N., Zhang, Z. H., Jadulco, R. C. et al. (2010). WLS-dependent secretion of WNT3A requires Ser209 acylation and vacuolar acidification. *Journal of Cell Science* **123**, 3357-3367.

Coudreuse, D. Y. M., Roel, G., Betist, M. C., Destree, O. and Korswagen, H. C. (2006). Wnt gradient formation requires retromer function in Wnt-producing cells. *Science* **312**, 921-924.

Dang, H., Li, Z., Skolnik, E. Y. and Fares, H. (2004). Disease-related myotubularins function in endocytic traffic in Caenorhabditis elegans. *Molecular Biology of the Cell* **15**, 189-196.

Di Paolo, G. and De Camilli, P. (2006). Phosphoinositides in cell regulation and membrane dynamics. *Nature* **443**, 651-657.

Fares, H. and Greenwald, I. (2001). Genetic analysis of endocytosis in Caenorhabditis elegans: Coelomocyte uptake defective mutants. *Genetics* **159**, 133-145.

Franch-Marro, X., Marchand, O., Piddini, E., Ricardo, S., Alexandre, C. and Vincent, J. P. (2005). Glypicans shunt the Wingless signal between local signalling and further transport. *Development* **132**, 659-666.

Franch-Marro, X., Wendler, F., Guidato, S., Griffith, J., Baena-Lopez, A., Itasaki, N., Maurice, M. M. and Vincent, J. P. (2008). Wingless secretion requires endosome-

to-Golgi retrieval of Wntless/Evi/Sprinter by the retromer complex. *Nature Cell Biology* **10**, 170-U40.

Fu, J., Jiang, M., Mirando, A. J., Yu, H. M. I. and Hsu, W. (2009). Reciprocal regulation of Wnt and Gpr177/mouse Wntless is required for embryonic axis formation. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 18598-18603.

Gagliardi, M., Piddini, E. and Vincent, J.-P. (2008). Endocytosis: A Positive or a Negative Influence on Wnt Signalling? *Traffic* **9**, 1-9.

Galli, L. M., Barnes, T. L., Secrest, S. S., Kadowaki, T. and Burrus, L. W. (2007). Porcupine-mediated lipid-modification regulates the activity and distribution of Wnt proteins in the chick neural tube. *Development* **134**, 3339-3348.

Goodman, R. M., Thombre, S., Firtina, Z., Gray, D., Betts, D., Roebuck, J., Spana, E. P. and Selva, E. M. (2006). Sprinter: a novel transmembrane protein required for Wg secretion and signaling. *Development* **133**, 4901-4911.

Greco, V., Hannus, M. and Eaton, S. (2001). Argosomes: A potential vehicle for the spread of morphogens through epithelia. *Cell* **106**, 633-645.

Harris, J., Honigberg, L., Robinson, N. and Kenyon, C. (1996). Neuronal cell migration in C-elegans: Regulation of Hox gene expression and cell position. *Development* **122**, 3117-3131.

Harterink, M., Kim, D. H., Middelkoop, T. C., Thang Dinh, D., van Oudenaarden, A. and Korswagen, H. C. (2011a). Neuroblast migration along the anteroposterior axis of C. elegans is controlled by opposing gradients of Wnts and a secreted Frizzled-related protein. *Development* **138**, 2915-2924.

Harterink, M. and Korswagen, H. C. (2012). Dissecting the Wnt secretion pathway: key questions on the modification and intracellular trafficking of Wnt proteins. *Acta Physiologica* **204**, 8-16.

Harterink, M., Port, F., Lorenowicz, M. J., McGough, I. J., Silhankova, M., Betist, M. C., van Weering, J. R. T., van Heesbeen, R., Middelkoop, T. C., Basler, K. et al. (2011b). A SNX3-dependent retromer pathway mediates retrograde transport of the Wnt sorting receptor Wntless and is required for Wnt secretion. *Nature Cell Biology* **13**, 914-U358.

Herman, M. A. (2001). C-elegans POP-1/TCF functions in a canonical Wnt pathway that controls cell migration acid in a noncanonical Wnt pathway that controls cell polarity. *Development* **128**, 581-590.

Herman, M. A., Vassilieva, L. L., Horvitz, H. R., Shaw, J. E. and Herman, R. K. (1995). The C. elegans gene *lin*-44, which controls the polarity of certain asymmetric cell divisions, encodes a Wnt protein and acts cell nonautonomously. *Cell* **83**, 101-110.

Hilliard, M. A. and Bargmann, C. I. (2006). Wnt signals and frizzled activity orient anterior-posterior axon outgrowth in C-elegans. *Developmental Cell* **10**, 379-390.

Hofmann, K. (2000). A superfamily of membrane-bound O-acyltransferases with implications for Wnt signaling. *Trends in Biochemical Sciences* **25**, 111-112.

Honigberg, L. and Kenyon, C. (2000). Establishment of left/right asymmetry in neuroblast migration by UNC-40/DCC, UNC-73/Trio and DPY-19 proteins in C.elegans. *Development* **127**, 4655-4668.

Höning, S., Ricotta, D., Krauss, M., Späte, K., Spolaore, B., Motley, A., Robinson, M., Robinson, C., Haucke, V. and Owen, D. J. (2005). Phosphatidylinositol-(4,5)-Bisphosphate Regulates Sorting Signal Recognition by the Clathrin-Associated Adaptor Complex AP2. *Molecular Cell* **18**, 519-531.

Horazdovsky, B. F., Davies, B. A., Seaman, M. N. J., McLaughlin, S. A., Yoon, S. and Emr, S. D. (1997). A sorting nexin-1 homologue, vps5p, forms a complex with vps17p and is required for recycling the vacuolar protein-sorting receptor. *Molecular Biology of the Cell* **8**, 1529-1541.

Ch'ng, Q., Williams, L., Lie, Y. S., Sym, M., Whangbo, J. and Kenyon, C. (2003). Identification of genes that regulate a left-right asymmetric neuronal migration in Caenorhabditis elegans. *Genetics* **164**, 1355-1367.

Ching, W., Hang, H. C. and Nusse, R. (2008). Lipid-independent secretion of a Drosophila Wnt protein. *Journal of Biological Chemistry* **283**, 17092-17098.

Jones, A. K. and Sattelle, D. B. (2004). Functional genomics of the nicotinic acetylcholine receptor gene family of the nematode, Caenorhabditis elegans. *Bioessays* **26**, 39-49.

Kadowaki, T., Wilder, E., Klingensmith, J., Zachary, K. and Perrimon, N. (1996). The segment polarity gene porcupine encodes a putative multitransmembrane protein involved in Wingless processing. *Genes & Development* **10**, 3116-3128.

Kamath, R. S., Fraser, A. G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M. et al. (2003). Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. *Nature* **421**, 231-237.

Kikuchi, A., Yamamoto, H. and Sato, A. (2009). Selective activation mechanisms of Wnt signaling pathways. *Trends in Cell Biology* **19**, 119-129.

Kim, H., Cheong, S. M., Ryu, J., Jung, H. J., Jho, E. H. and Han, J. K. (2009). Xenopus Wntless and the Retromer Complex Cooperate To Regulate XWnt4 Secretion. *Molecular and Cellular Biology* **29**, 2118-2128.

Komekado, H., Yamamoto, H., Chiba, T. and Kikuchi, A. (2007). Glycosylation and palmitoylation of Wnt-3a are coupled to produce an active form of Wnt-3a. *Genes to Cells* **12**, 521-534.

Korkut, C., Ataman, B., Ramachandran, P., Ashley, J., Barria, R., Gherbesi, N. and Budnik, V. (2009). Trans-Synaptic Transmission of Vesicular Wnt Signals through Evi/Wntless. *Cell* **139**, 393-404.

Korswagen, H. C. (2002). Canonical and non,canonical Wnt signaling pathways in Calenorhabditis elgans: variations on a common signaling theme. *Bioessays* **24**, 801-810.

Korswagen, H. C., Coudreuse, D. Y. M., Betist, M. C., van de Water, S., Zivkovic, D. and Clevers, H. C. (2002). The Axin-like protein PRY-1 is a negative regulator of a canonical Wnt pathway in C-elegans. *Genes & Development* **16**, 1291-1302.

Korswagen, H. C., Herman, M. A. and Clevers, H. C. (2000). Distinct beta-catenins mediate adhesion and signalling functions in C-elegans. *Nature* **406**, 527-532.

Kurayoshi, M., Yamamoto, H., Izumi, S. and Kikuchi, A. (2007). Post-translational palmitoylation and glycosylation of Wnt-5a are necessary for its signalling. *Biochemical Journal* **402**, 515-523.

Laporte, J., Bedez, F., Bolino, A. and Mandel, J. L. (2003). Myotubularins, a large disease-associated family of cooperating catalytically active and inactive phosphoinositides phosphatases. *Human Molecular Genetics* **12**, R285-R292.

Logan, C. Y. and Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annual Review of Cell and Developmental Biology* **20**, 781-810.

Maloof, J. N., Whangbo, J., Harris, J. M., Jongeward, G. D. and Kenyon, C. (1999). A Wnt signaling pathway controls Hox gene expression and neuroblast migration in C-elegans. *Development* **126**, 37-49.

Mulligan, K. A., Fuerer, C., Ching, W., Fish, M., Willert, K. and Nusse, R. (2012). Secreted Wingless-interacting molecule (Swim) promotes long-range signaling by maintaining Wingless solubility. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 370-377.

Nathoo, A. N., Moeller, R. A., Westlund, B. A. and Hart, A. C. (2001). Identification of neuropeptide-like protein gene families in Caenorhabditis elegans and other species. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 14000-14005.

Nusse, R. (2008). Wnt signaling and stem cell control. *Cell Research* **18**, 523-527.

Oosterveen, T., Couldreuse, D. Y. M., Yang, P.-T., Fraser, E., Bergsma, J., Dale, T. C. and Korswagen, H. C. (2007). Two functionally distinct Axin-like proteins regulate canonical Wnt signaling in C-elegans. *Developmental Biology* **308**, 438-448.

Pan, C. L., Baum, P. D., Gu, M. Y., Jorgensen, E. M., Clark, S. G. and Garriga, G. (2008a). C-elegans AP-2 and retromer control Wnt signaling by regulating MIG-14/Wntless. *Developmental Cell* **14**, 132-139.

Pan, C. L., Howell, J. E., Clark, S. G., Hilliard, M., Cordes, S., Bargmann, C. I. and Garriga, G. (2006). Multiple Wnts and frizzled receptors regulate anteriorly directed cell and growth cone migrations in Caenorhabditis elegans. *Developmental Cell* **10**, 367-377.

Pan, W. J., Choi, S. C., Wang, H., Qin, Y. B., Volpicelli-Daley, L., Swan, L., Lucast, L., Khoo, C., Zhang, X. W., Li, L. et al. (2008b). Wnt3a-mediated formation of phosphatidylinositol 4,5-bisphosphate regulates LRP6 phosphorylation. *Science* **321**, 1350-1353.

Panakova, D., Sprong, H., Marois, E., Thiele, C. and Eaton, S. (2005). Lipoprotein particles are required for Hedgehog and Wingless signalling. *Nature* **435**, 58-65.

Park, S. K., Link, C. D. and Johnson, T. E. (2010). Life-span extension by dietary restriction is mediated by NLP-7 signaling and coelomocyte endocytosis in C. elegans. *Faseb Journal* **24**, 383-392.

Park, S. K., Tedesco, P. M. and Johnson, T. E. (2009). Oxidative stress and longevity in Caenorhabditis elegans as mediated by SKN-1. *Aging Cell* **8**, 258-269.

Patton, A., Knuth, S., Schaheen, B., Dang, H., Greenwald, I. and Fares, H. (2005). Endocytosis function of a ligand-gated ion channel homolog in Caenorhabditis elegans. *Current Biology* **15**, 1045-1050.

Polakis, P. (2007). The many ways of Wnt in cancer. *Current Opinion in Genetics & Development* **17**, 45-51.

Port, F. and Basler, K. (2010). Wnt Trafficking: New Insights into Wnt Maturation, Secretion and Spreading. *Traffic* **11**, 1265-1271.

Port, F., Kuster, M., Herr, P., Furger, E., Banziger, C., Hausmann, G. and Basler, K. (2008). Wingless secretion promotes and requires retromer-dependent cycling of Wntless. *Nature Cell Biology* **10**, 178-U48.

Prasad, B. C. and Clark, S. G. (2006). Wnt signaling establishes anteroposterior neuronal polarity and requires retromer in C-elegans. *Development* **133**, 1757-1766.

Rocheleau, C. E., Downs, W. D., Lin, R. L., Wittmann, C., Bei, Y. X., Cha, Y. H., Ali, M., Priess, J. R. and Mello, C. C. (1997). Wnt signaling and an APC-related gene specify endoderm in early C-elegans embryos. *Cell* **90**, 707-716.

Salser, S. J. and Kenyon, C. (1992). Activation of a C. elegans Antennapedia homolog in migrating cells controls their direction of migration. *Nature* **355**, 255-258.

Sawa, H., Lobel, L. and Horvitz, H. R. (1996). The Caenorhabditis elegans gene lin-17, which is required for certain asymmetric cell divisions, encodes a putative protein similar to seven-transmembrane protein similar to the Drosophila Frizzle protein. *Genes & Development* **10**, 2189-2197.

Seaman, M. N. J., Marcusson, E. G., Cereghino, J. L. and Emr, S. D. (1997). Endosome to Golgi retrieval of the vacuolar protein sorting receptor, Vps10p, requires the function of the VPS29, VPS30, and VPS35 gene products. *Journal of Cell Biology* **137**, 79-92.

Seaman, M. N. J., McCaffery, J. M. and Emr, S. D. (1998). A membrane coat complex essential for endosome-to-Golgi retrograde transport in yeast. *Journal of Cell Biology* **142**, 665-681.

Shackleford, G. M., Shivakumar, S., Shiue, L., Mason, J., Kenyon, C. and Varmus, H. E. (1993). Two Wnt genes in Caenorhabditis elegans. *Oncogene* **8**, 1857-1864.

Silhankova, M. and Korswagen, H. C. (2007). Migration of neuronal cells along the anterior-posterior body axis of C. elegans: Wnts are in control. *Current Opinion in Genetics & Development* **17**, 320-325.

Silhankova, M., Port, F., Harterink, M., Basler, K. and Korswagen, H. C. (2010). Wnt signalling requires MTM-6 and MTM-9 myotubularin lipid-phosphatase function in Wnt-producing cells. *Embo Journal* **29**, 4094-4105.

Song, H. O., Lee, J., Ji, Y. J., Dwivedi, M., Cho, J. H., Park, B. J. and Ahnn, J. (2010). Calcineurin Regulates Coelomocyte Endocytosis via DYN-1 and CUP-4 in Caenorhabditis elegans. *Molecules and Cells* **30**, 255-262.

Taelman, V. F., Dobrowolski, R., Plouhinec, J. L., Fuentealba, L. C., Vorwald, P. P., Gumper, I., Sabatini, D. D. and De Robertis, E. M. (2010). Wnt Signaling Requires Sequestration of Glycogen Synthase Kinase 3 inside Multivesicular Endosomes. *Cell* **143**, 1136-1148.

Takada, R., Satomi, Y., Kurata, T., Ueno, N., Norioka, S., Kondoh, H., Takao, T. and Takada, S. (2006). Monounsaturated fatty acid modification of Wnt protein: Its role in Wnt secretion. *Developmental Cell* **11**, 791-801.

Tanaka, K., Okabayashi, K., Asashima, M., Perrimon, N. and Kadowaki, T. (2000). The evolutionarily conserved porcupine gene family is involved in the processing of the Wnt family. *European Journal of Biochemistry* **267**, 4300-4311.

Tanneberger, K., Pfister, A. S., Brauburger, K., Schneikert, J., Hadjihannas, M. V., Kriz, V., Schulte, G., Bryja, V. and Behrens, J. (2011). Amer1/WTX couples Wnt-induced formation of PtdIns(4,5)P-2 to LRP6 phosphorylation. *Embo Journal* **30**, 1433-1443.

Taylor, G. S., Maehama, T. and Dixon, J. E. (2000). Myotubularin, a protein tyrosine phosphatase mutated in myotubular myopathy, dephosphorylates the lipid second messenger, phosphatidylinositol 3-phosphate. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 8910-8915.

Ten Berge, D., Kurek, D., Blauwkamp, T., Koole, W., Maas, A., Eroglu, E., Siu, R. K. and Nusse, R. (2011). Embryonic stem cells require Wnt proteins to prevent differentiation to epiblast stem cells. *Nature Cell Biology* **13**, 1070-U88.

Thorpe, C. J., Schlesinger, A., Carter, J. C. and Bowerman, B. (1997). Wnt signaling polarizes an early C-elegans blastomere to distinguish endoderm from mesoderm. *Cell* **90**, 695-705.

Verges, M., Luton, F., Gruber, C., Tiemann, F., Reinders, L. G., Huang, L., Burlingame, A. L., Haft, C. R. and Mostov, K. E. (2004). The mammalian retromer regulates transcytosis of the polymeric immunoglobulin receptor. *Nature Cell Biology* **6**, 763-769.

Walston, T., Guo, C. B., Proenca, R., Wu, M. F., Herman, M., Hardin, J. and Hedgecock, E. (2006). mig-5/Dsh controls cell fate determination and cell migration in C-elegans. *Developmental Biology* **298**, 485-497.

Whangbo, J., Harris, J. and Kenyon, C. (2000). Multiple levels of regulation specify the polarity of an asymmetric cell division in C.elegans. *Development* **127**, 4587-4598.

Whangbo, J. and Kenyon, C. (1999). A Wnt signaling system that specifies two patterns of cell migration in C-elegans. *Molecular Cell* **4**, 851-858.

Willert, K., Brown, J. D., Danenberg, E., Duncan, A. W., Weissman, I. L., Reya, T., Yates, J. R. and Nusse, R. (2003). Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* **423**, 448-452.

Xue, Y. Z., Fares, H., Grant, B., Li, Z., Rose, A. M., Clark, S. G. and Skolnik, E. Y. (2003). Genetic analysis of the myotubularin family of phosphatases in Caenorhabditis elegans. *Journal of Biological Chemistry* **278**, 34380-34386.

Yang, P. T., Lorenowicz, M. J., Silhankova, M., Coudreuse, D. Y. M., Betist, M. C. and Korswagen, H. C. (2008). Wnt signaling requires retromer-dependent recycling of MIG-14/Wntless in Wnt-producing cells. *Developmental Cell* **14**, 140-147.

Zhai, L., Chaturvedi, D. and Cumberledge, S. (2004). Drosophila Wnt-1 undergoes a hydrophobic modification and is targeted to lipid rafts, a process that requires porcupine. *Journal of Biological Chemistry* **279**, 33220-33227.

Zinovyeva, A. Y. and Forrester, W. C. (2005). The C-elegans Frizzled CFZ-2 is required for cell migration and interacts with multiple Writ signaling pathways. *Developmental Biology* **285**, 447-461.

Zinovyeva, A. Y., Yamamoto, Y., Sawa, H. and Forrester, W. C. (2008). Complex network of Wnt signaling regulates neuronal migrations during Caenorhabditis elegans development. *Genetics* **179**, 1357-1371.